GENETIC BASIS
OF
INHERITED EYE DISEASES

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Chapter 1

General introduction

The eye is a vital organ of the human body as vision is the basic necessity of a human being. Interestingly, a significant portion of the human brain is involved in interpreting the visual signals received in the form of nerve impulses from the photosensitive cells of the eye.[1] A diseased eye cannot perform its function properly and thus vision is impaired, which subsequently results in the disability of the affected individual. Unlike in the western world, in the third world, affected individuals are thought to be a burden on the family because they cannot cope with the hardships of life, socially as well as economically.

Visual impairment that results from minor refractive errors can usually be corrected using artificial lenses but in other cases usually the loss of vision is irreversible and cannot be treated. Diseases that result in irreversible loss of vision can be sub-divided into two groups; traumatic eye diseases resulting from injuries and inherited eye diseases (either monogenic or multifactorial). Traumatic injuries may lead to blindness if they are severe enough and sometimes even minor injuries, if not treated properly, may end up in a major dystrophy. Similarly, genetic mutations may result in apoptosis-mediated cellular death. Before going into the details of eye diseases an overview of the anatomical features of the eye is called for.

1.1. Anatomical structure of the human eye

Almost all vertebrate species have a pair of eyes situated in the skull below the forehead. The human eye is a semi-spherical ball-like structure composed of three layers; the outer layer is the sclera, the middle layer is the choroid and the innermost layer is the retina (Figure 1.1). The transparent portion of the sclera in front of the eye is known as the cornea. Next to the lens, the cornea is partially responsible for the refraction of light. Behind the cornea there is a ring-like structure, the iris, which contains a central opening, the pupil. The latter contracts in response to the intensity of the incoming light, and thus controls the amount of light entering into the lumen of the eye. The lumen between the cornea and iris is filled with aqueous humor, while the lens is situated posterior to the iris. Ciliated muscles hold the lens in its proper place; these muscles are also involved in focusing the lens for near and distant vision. Posterior to the lens is the interior chamber of the eye, which is filled with a viscous vitreous humor. The innermost part of the eye is the retina, which is involved in image processing. The central part of retina is the macula where the images are formed. The center of the macula, which is responsible for colour vision and high acuity vision, is the fovea. The fovea is the cone-rich region of the retina and contains a trough in the center to allow maximal penetration of light that can trigger the densely packed cone photoreceptors.

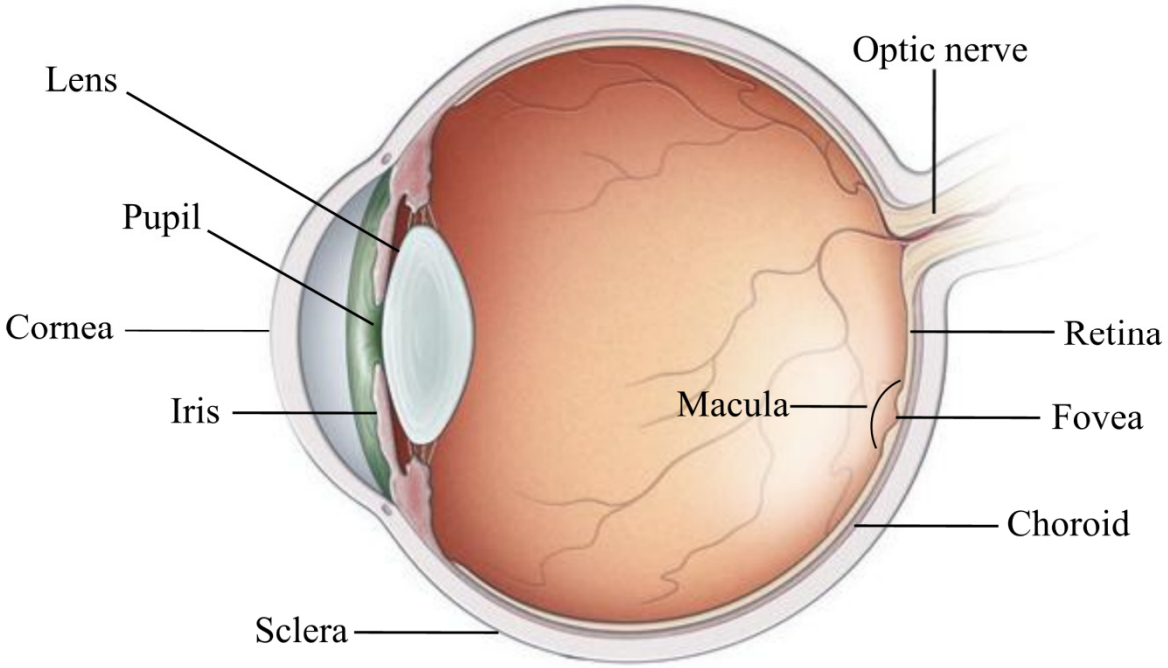

Figure 1.1 Structure of the human eye (adapted and modified from Jacobson and Cideciyan[2]).

1.1.1. The retina

The innermost and multi-layered structure of the eye, which contains the photoreceptor cells responsible for image processing, is the retina. In 1583 Felix Plater was the first person who described the retina as a target tissue for the incoming light.[3] The retina is further divided into different layers, which include an outer layer the retinal pigment epithelium (RPE), followed by an outer segment layer, an outer limiting membrane, an outer nuclear layer, an outer plexiform layer, an inner nuclear layer, an inner plexiform layer, a ganglion cell layer, an optic nerve fiber layer, and an inner limiting membrane (Figure 1.2). Of these, the RPE consists of a single layer of hexagonal cells that separates the neural retina from the choroid. The RPE, among many other functions, is involved in supplying nutrients to the other internal layers of the retina.[4] In addition, the RPE has a pigment, melanin, which helps in the absorption of reflected light that is not used by the photoreceptor cells. The RPE is also involved in the phagocytosis of shed outer segments of the photoreceptors. Next to the RPE is the photoreceptor layer consisting of rods and cones, with their outer segments embedded in the RPE, and their cellular bodies being present in the outer nuclear layer. Axons of the rods, the cones, and their associated bipolar and horizontal cells, which are present in the inner nuclear layer, exchange synaptic signals in the outer plexiform layer. The inner nuclear layer also harbours amacrine and Müller glial cells. Bipolar cells transfer signals to the amacrine

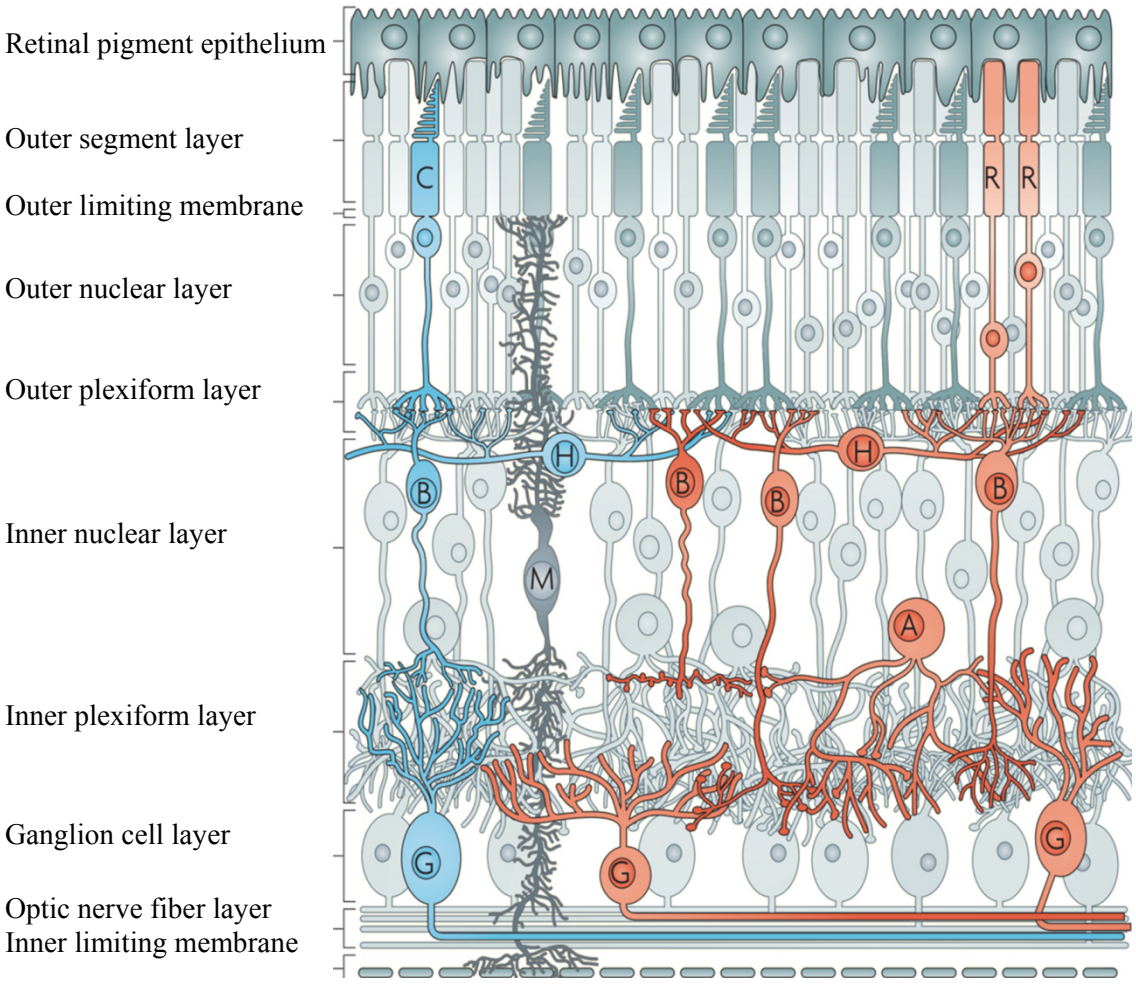

Figure 1.2 Arrangement of retinal layers. The retina consists of ten distinct layers. The outermost layer is the retinal pigment epithelium (RPE), into which outer segments of rod (R) and cone (C) photoreceptors are embedded. Next to the RPE is the layer that contains the outer segments of the rod and cone photoreceptors, whereas their cell bodies constitute the outer nuclear layer. The axons of photoreceptors terminate in the outer plexiform layer. The inner nuclear layer contains horizontal cells (H), bipolar cells (B), Müller cells (M) and amacrine cells (A). The inner plexiform layer consists of the synaptic terminals of bipolar, amacrine and ganglion cells (G). The ganglion cell layer lies between the inner plexiform layer and optic nerve fiber layer. Next to the ganglion cell layer is the optic nerve fiber layer. Inner and outer limiting membranes contain the endings of Müller glial cells (adapted and modified from Swaroop *et al.*[5]).

and ganglion cells via the synapses in the inner plexiform layer. The ganglion cells transfer the signal to the brain through the optic nerve. The inner and outer limiting membranes contain endings of the Müller glia cells.[5] Input from the retina is sent to the brain where the midbrain controls different parameters of the visual process including eye movements, pupil size, and circadian photoentrainment, in response to the signals received from the neuronal retinal network of cells.[6] Finally, the retinal information reaches the visual cortex, where it results in the interpretation of the signals by the brain.[7]

1.1.1.1. Photoreceptors

One of the most common etiologies of blindness worldwide is the degeneration of photoreceptor cells.[8] Photoreceptors are light sensitive modified-cilia like bodies, which are involved in the visual signal transduction. These cells differentiate at later stages of retinal development just before the Müller glial cells.[9] In the process of vision the signal is received in the form of photons that enter the eye through the cornea, pass through the aqueous humor of the anterior chamber, cross the lens, enter into the vitreous and finally excite the photoreceptors in the retina.

Max Schultze in 1866 described photoreceptors as functional entities that are involved in the vision process and named them rods and cones,[10] which have diverse structures and functions in different living organisms.[11] The rod photoreceptors are of one type only, whereas three different types of cones are used to differentiate between different colours.[12]

Rod photoreceptors

The rod photoreceptors (Figure 1.3) are highly sensitive to light as compared to the cone photoreceptors[13] and have the ability to detect even a single photon of light.[14] This remarkable light sensing ability is ideal for low light conditions.[12] Moreover, the number of rod cells (approximately 125 million) is much higher than the number of cones (approximately 7 million).

The rod photoreceptors are mostly responsible for night vision and remain inactive during bright light conditions.[15] Recovery of the sensitivity phase, known as dark adaptation, of rod photoreceptors is relatively slower than that of the cone photoreceptors and may take up to an hour.[16,17]

Cone photoreceptors

The cone photoreceptors (Figure 1.3) are concentrated in the macula and are more densely packed in the foveal region. The cone photoreceptors are mainly responsible for colour vision and visual acuity during day time and under bright light conditions. There are three types of cone cells, red, green and blue, based on the presence of different photopigments. Red cones respond to long wavelengths of 564-580 nm, green cones respond to medium wavelengths of 534-545 nm and blue cones respond to short wavelengths of 420-440 nm, and are thus used to discriminate among the different colours. The cone photoreceptors are mostly functional

during bright light conditions and the light sensing ability of the cone photoreceptors is almost one hundred times less than that of the rod photoreceptors.[18,19] Because of this much lower sensitivity, cone photoreceptors can easily adapt to varying intensities of light and remain light sensitive during the entire day time period, relaying messages to the brain.[12,20]

1.2. Mechanism of vision

The process of vision in the rods begins with the photo-isomerization of the opsin-bound chromophore (rhodopsin) in the outer segment of the photoreceptors.[7] The photo-activated visual pigment rhodopsin then initiates a cascade of reactions, which result in the closure of the cationic ion channels situated in the plasma membrane, leading to hyperpolarization of the cells.[11] The mechanism of vision can be divided into two distinct steps, the phototransduction and the retinoid cycle.

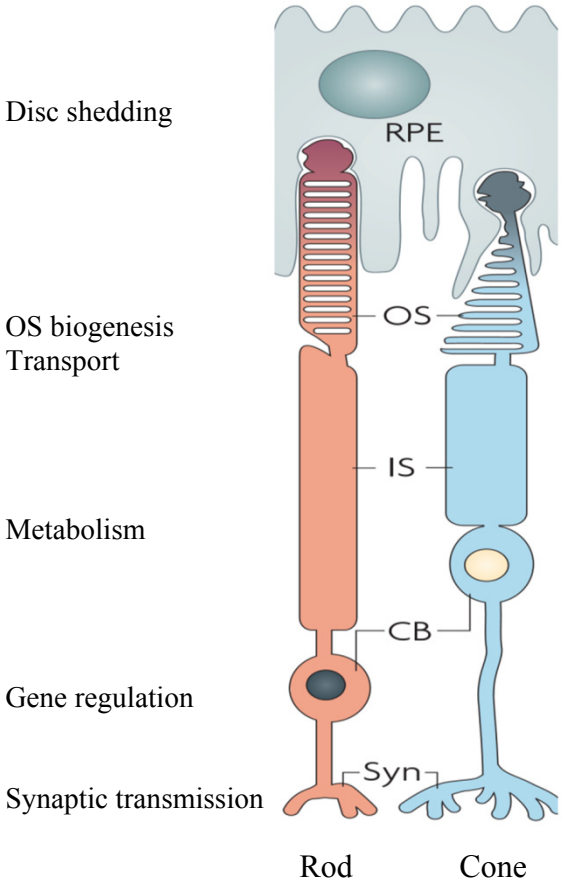

Figure 1.3 Structure of rod and cone photoreceptors. Rod and cone outer segments (OS) are embedded in the retinal pigment epithelium (RPE). Inner segments (IS) are mainly involved in metabolic processes. Cell bodies (CB) contain the nucleus and are involved in gene regulation. Synaptic transmission takes place through the synapses (Syn) of rods and cones (adapted and modified from Swaroop *et al.*[5]).

1.2.1. Mechanism of phototransduction

The phototransduction cascade takes place in the photoreceptor outer segments (OS) where light absorption results in the photo-isomerization of the opsin-bound chromophore 11-*cis* retinal to all-*trans* retinal, and activation of the visual pigment rhodopsin (Figure 1.4).[7,21] This activated rhodopsin then catalyzes the exchange of transducin-bound guanosine diphosphate (GDP) by guanosine triphosphate (GTP) leading to activation of transducin,[21-23] which is a heterotrimeric G protein. Upon activation of transducin, the GTP-bound alpha subunit dissociates from the other two subunits exposing its active site, which subsequently binds to and activates the cyclic guanosine monophosphate (cGMP) phosphodiesterase. The cGMP phosphodiesterase hydrolyzes cGMP to 5'GMP resulting in the intracellular depletion of cGMP levels, which then triggers the closure of cyclic nucleotide gated (CNG) channels. Closure of CNG channels finally leads to the hyperpolarization of the photoreceptors, which results in a decrease in synaptic release of the neurotransmitter glutamate.[7,24-26]

After hyperpolarization, the rhodopsin recovery phase begins with its phosphorylation by rhodopsin kinase and binding of arrestin.[21] The level of cGMP is also restored with the help of guanylyl cyclase (GC) that is activated by calcium-binding protein guanylyl cyclase activating protein (GCAP). This GC-mediated increase in cGMP level subsequently results in the opening of the CNG channel due to which the cell becomes depolarized again. Opening of CNG channel results in the elevation of intracellular Ca^{2+}, which results in the inactivation of GC. Intracellular Ca^{2+} levels are regulated through the Na^{+}/Ca^{2+}-K^{+} exchanger that is present in the plasma membrane.[27]

1.2.2. Retinoid cycle

The rod specific retinoid cycle occurs in the rod outer segment and RPE (Figure 1.5). In the rod photoreceptor OS, arrestin binding to rhodopsin results in the dissociation of all-*trans* retinal into the disc membranes of the rod photoreceptor. From the disc membranes of the outer segment, ATP binding cassette subfamily A member 4 (ABCA4) transports all-*trans* retinal to the cytoplasmic space, where it is subsequently reduced into the all-*trans* retinol by retinol dehydrogenase 12 (RDH12). From the cytoplasmic space all-*trans* retinol is released into the interphotoreceptor matrices with the help of interphotoreceptor binding protein (IRBP). All-*trans* retinol binds cellular retinol-binding protein-1 (CRALBP1) and diffuses into the RPE where it is converted into all-*trans* retinyl ester by lecithin retinol acyl transferase (LRAT). All-*trans* retinyl ester is then converted to 11-*cis* retinol by RPE-specific

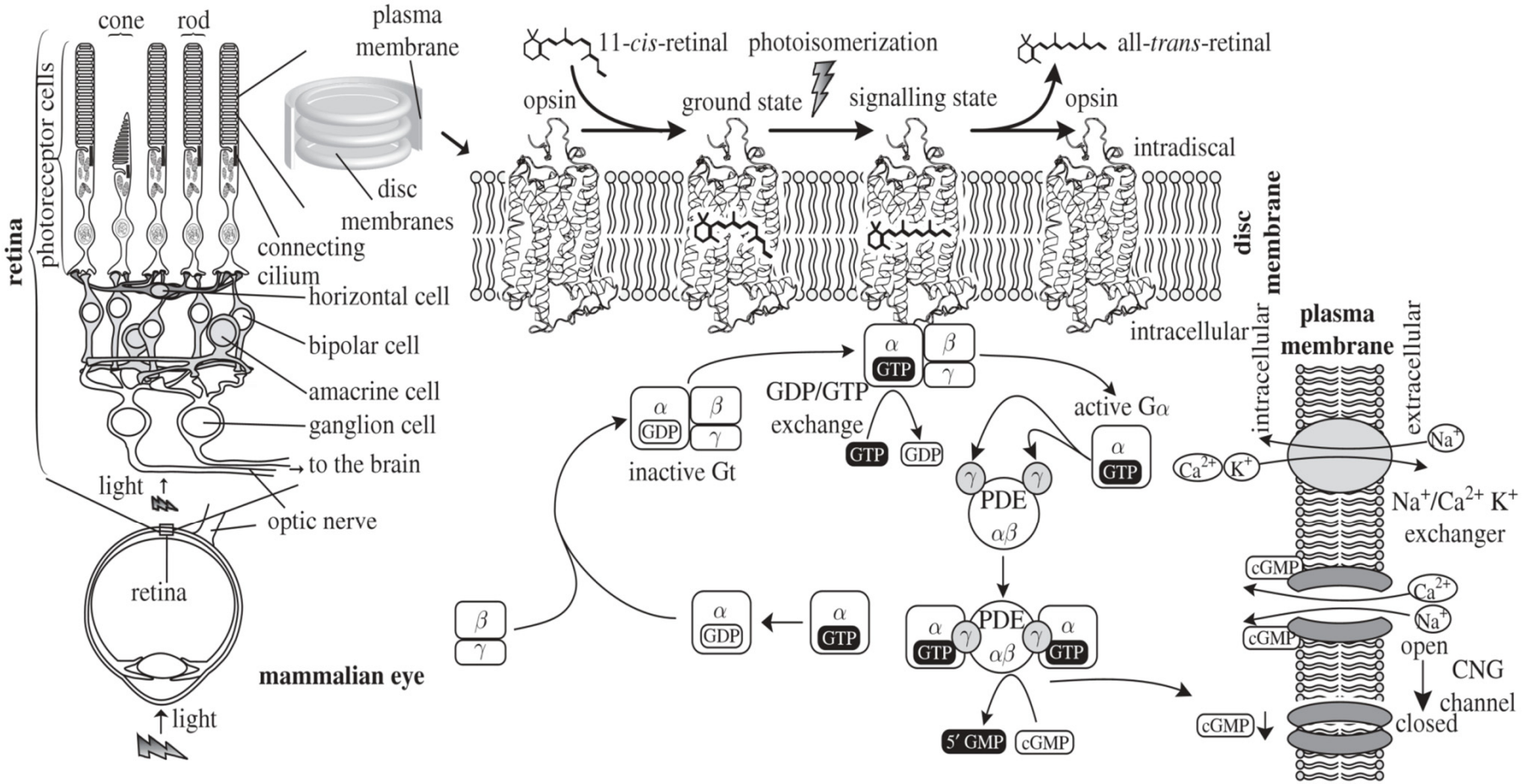

Figure 1.4 Mechanism of mammalian phototransduction from light perception to hyperpolarization of the photoreceptors. Photoisomerization of 11-*cis* retinal to all-*trans* retinal starts a cascade of reactions leading to the hyperpolarization of the cell. Different intracellular signaling molecules and membrane channels including guanosine diphosphate (GDP), guanosine triphosphate (GTP), phosphodiesterase (PDE), cyclic guanosine monophosphate (cGMP), 5' guanosine monophosphate (5'GMP), cyclic nucleotide gated (CNG) channel and sodium/calcium and potassium (Na^+/Ca^{2+} K^+) exchangers take part in the whole process (adapted from Shichida and Matsuyama[21]).

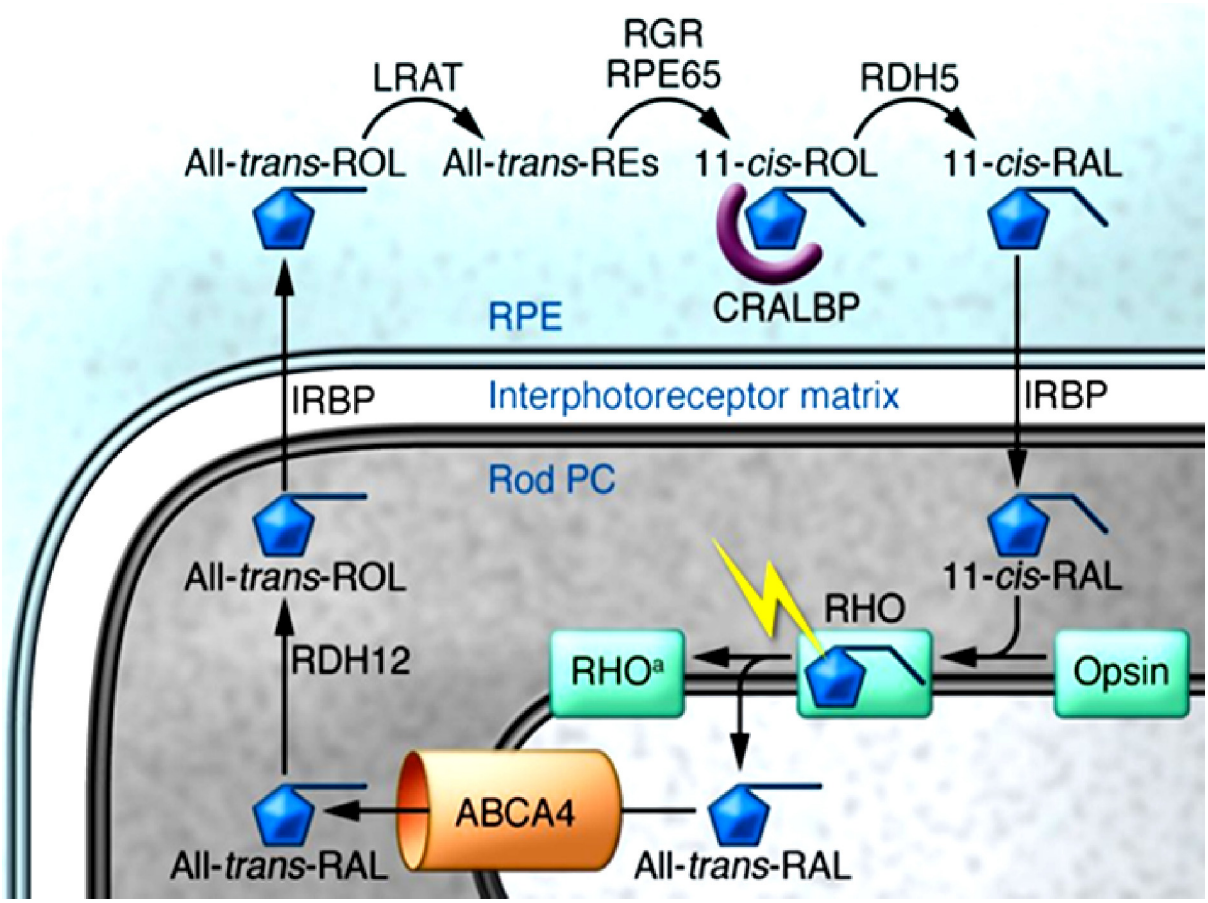

Figure 1.5 Retinoid cycle showing the regeneration of the visual chromophore 11-*cis* retinal in the RPE by the action of different stereospecific enzymes. In the rod photoreceptor cell (PC) outer segment 11-*cis* retinal (11-*cis*-RAL) is converted to all-*trans* retinol (all-*trans*-ROL) by the action of different enzymes. Rhodopsin (RHO) is a chromophore bound photosensitive membrane protein that is activated by photoisomerization of 11-*cis* RAL to all-*trans* RAL. ATP binding cassette subfamily A member 4 (ABCA4) is required for chromophore transport into the cytoplasm. Retinal dehydrogenase 12 (RDH12) is a stereospecific enzyme present in the photoreceptor outer segment. Interphotoreceptor binding protein (IRBP) is present in interphotoreceptor matrix. In the retinal pigment epithelium (RPE) several stereospecific enzymes including lecithin retinol acyl transferase (LRAT), retinal pigment epithelium specific 65 kDa protein (RPE65), retinal G protein coupled receptor (RGR), 11-*cis* retinol dehydrogenase (RDH5) and cellular retinaldehyde binding protein (CRALBP) are involved in the regeneration of the chromophore (adapted from den Hollander *et al*.[30]).

65 kDa protein (RPE65) that is an isomerase, the activity of which is thought to be controlled by a non-photoreceptor opsin known as the retinal G protein coupled receptor (RGR).[28] The 11-*cis* retinol dehydrogenase (RDH5) catalyzes the oxidation of 11-*cis* retinol into 11-*cis* retinal, which is then transported back into the interphotoreceptor matrices by binding with IRBP. From the interphotoreceptor matrices 11-*cis* retinal diffuses into rod photoreceptor outer segment where it binds to the opsin to take part in another round of phototransduction.[29-31]

In the rod photoreceptors the conversion of the all-*trans* retinol to 11-*cis* retinal takes place in the RPE, whereas in the cone photoreceptor cells this conversion is not fully dependent on the RPE, and this part of the retinoid cycle also takes place in the Müller cells.[32]

1.3. Inherited eye diseases

Monogenic retinal diseases are relatively rare with an estimated worldwide prevalence of 1/2000 persons.[33] In comparison, the multifactorial eye diseases glaucoma and age-related macular degeneration affect 2% and 1.5% of the population, respectively.[34] The monogenic retinal diseases often are progressive and affect young persons, and thereby have a significant negative impact on the quality of life of the affected persons and represent a huge economic burden on the society. These retinal anomalies may involve different cell types of the retina including photoreceptors and the RPE. Based upon which cell types degenerate first, retinal dystrophies can be further classified into cone dystrophy, cone-rod dystrophy or rod-cone dystrophy. In addition, several other features including age of onset and clinical phenotypes are also considered.

1.3.1. Non-syndromic eye diseases

Isolated eye disease phenotypes without any other anomaly are known as non-syndromic, which are mostly monogenic but rare digenic forms and mitochondrial inheritances have also been reported. Retinitis pigmentosa is a common example of a genetically inherited non-syndromic eye disease. Below, only the retinal phenotypes that were studied in this thesis are described.

1.3.1.1. Retinitis pigmentosa

The Dutch ophthalmologist F.C. Donders was the first person who used the term "retinitis pigmentosa" (RP) in 1857,[2] which is described as a heterogeneous disorder, both clinically as well as genetically, and is one of the most frequent monogenic retinal diseases worldwide with a prevalence of 1 in 3500 to 4000.[35,36] Clinically, RP is characterized by night blindness and an initial progressive loss of mid-peripheral vision, which further expands towards the periphery and central parts of the retina[37,38] leading to legal blindness in the mid-forties and ultimately can lead to complete blindness.[39,40] In RP a rod-cone pattern of photoreceptor degeneration is seen, in which the rod photoreceptor degeneration precedes cone degeneration.[34]

The diagnosis of RP mainly depends on two basic evaluations, fundus examination and measurement of electrophysiological responses of rods and cones. Fundus examination of the RP patient mostly reveals the presence of bone spicules in the peripheral region of the retina and attenuation of the retinal blood vessels, whereas measurement of electrophysiological responses of rods and cones help in assessing the health of the different types of photoreceptors. At early stages, RP is diagnosed by night blindness and mild attenuation of retinal vessels, because bone spicules mostly develop at later stages of the disease. Sometimes when patients with normal fundi complain about night blindness and there are no other symptoms of the disease then the disease is diagnosed as RP *sine pigmento*.[35]

The inheritance pattern of RP includes autosomal dominant (15-20% of cases), autosomal recessive (20-25%), and X-linked (10-15%), whereas isolated RP accounts for 40-55% of RP cases.[41] RP is mostly inherited as a single gene disorder[34] but digenic forms[42] and rare mitochondrial inheritance patterns have also been observed.[43]

The heterogeneous nature of RP is reflected by the growing number of causative genes, which determine the molecular etiology of the disease. Eight disease-linked loci and 55 different genes have been implicated in non-syndromic RP (https://sph.uth.tmc.edu/retnet/).

1.3.1.2. Fundus albipunctatus

Lauber described the fleck retina disease fundus albipunctatus (FA) for the first time in 1910.[44] FA is a rare genetically inherited condition and is considered a sub-type of congenital stationary night blindness (CSNB). Clinical symptoms of FA include the presence of yellowish white spots or flecks in the mid-peripheral region of the retina or scattered throughout the fundus and delayed dark adaptation.[45] In some cases of FA, cone dystrophy and macular degeneration have also been observed along with the presence of white dots but the degree of severity in such cases is highly variable.[46-48]

Symptoms of FA overlap with those of retinitis punctata albescens (RPA), which is another form of retinal disease. Common features of both conditions are the presence of yellowish white spots on the retina and loss of night vision.[49] FA is a stationary disease whereas RPA is progressive in nature. The only way to distinguish between these two is to measure the electrophysiological responses of rod and cone photoreceptor cells. The rod photoreceptors of FA patients take more than three hours to attain their normal state during the recovery phase while the rod photoreceptors of RPA patients fail to recover al all.[45] Alternatively, a regular

follow up examination of patients presenting with white spots on the fundus and normal visual acuity may be helpful in establishing the correct diagnosis.

1.3.2. Syndromic eye diseases

The presence of additional clinical features outside the eye is termed as syndromic forms of the disease. The most common syndromic eye disorder is Usher syndrome in which deafness and RP occur at the same time.[50] Another syndromic form of retinal dystrophy is Bardet-Biedl syndrome (BBS),[51,52] which is a highly complex ciliary disease involving many organs. As BBS was part of this thesis, more details of this disease follow below.

1.3.2.1. Bardet-Biedl syndrome

BBS is clinically characterized by the presence of four out of six primary characteristics, which include rod-cone dystrophy, polydactyly, obesity, intellectual disability, hypogonadism and renal malformation. In cases where three of these primary features are present, the diagnosis of BBS can only be established when at least two additional secondary features are also present. Secondary features include liver anomalies, diabetes mellitus, reproductive and developmental disorders, growth retardation, speech delays, and cardiovascular malfunctions.[51]

The inheritance pattern of BBS is mostly autosomal recessive, whereas digenic forms with tri-allelic inheritance have also been identified.[53-56] Genetic modifiers play an important role in modifying the disease phenotype in BBS patients.[57] Modifying effects of a third allele have been studied for BBS and a protective role of other alleles has also been elucidated.[58,59]

The prevalence of BBS is variable in different world populations, in the Faroe Islands it is 1 in 3,700, which is the highest in the world,[60] whereas in Switzerland BBS is prevalent at 1 in 160,000 individuals.[61]

Mutations in 17 different genes have been shown to be associated with BBS[62] pointing to the genetic heterogeneity of the disease. Most BBS genes express proteins, which are part of a protein complex known as the BBSome, which include BBS1, BBS2, BBS4, BBS5, BBS7, BBS8 and BBS9. Although the BBSome complex is thought to be involved in the biogenesis of the ciliary membrane and membrane trafficking across the primary cilium, its exact functions are not yet completely understood.[63]

1.4. Techniques used to identify the genetic basis of eye diseases

Presently, different gene mapping techniques are in use to identify the mutated genes in patients with inherited disorders. Selection of the gene mapping technique usually depends on two factors, i.e., pedigree structure and disease phenotype. When the pedigree analysis reveals no ambiguity about the inheritance pattern and the clinical phenotype has also been properly diagnosed then the next step is the selection of a suitable gene localization strategy.

1.4.1. Sanger sequencing of candidate genes

Sanger sequencing is considered the gold standard in determining the aberrations in the deoxyribonucleic acid (DNA) sequence because of its nucleotide level resolution.[64] The principle is based on the incorporation of dideoxynucleotides when copying a single-stranded DNA molecule, which results in random termination of the chain and the generation of fragments of different sizes. The fluorescently labelled fragments are then electrophoretically separated, fluorescence induced by a laser scanner and detected by a diode array.

Candidate gene sequencing by the Sanger method is a useful strategy when the disease is associated with an obvious causative gene. This is the case in e.g. familial hypercholesterolemia, for which the low density lipoprotein receptor gene (*LDLR*) is the most commonly mutated gene,[65] so it is convenient to first analyse *LDLR* by sequence analysis, instead of using other expensive techniques as a first step. Similarly, persons with RP with a perifoveal annular ring in the fundus need to be screened first for tubby like protein 1 (*TULP1*) mutations, and patients with FA should initially be tested for *RDH5* mutations.[66,67] Although Sanger sequencing is the gold standard it cannot be used for large scale sequencing projects due to its lack of cost-effectiveness.

1.4.2. Linkage analysis

If the clinical phenotype in a family does not directly point towards the involvement of a specific gene, and mutations in one of many genes need to be considered, linkage analysis can be used to determine genomic regions that are shared by affected individuals within a family but are different in non-affected relatives. Linkage analysis is a classic genetic mapping technique commonly used to map disease associated loci and genes.[68] Establishment of genetic linkage primarily depends on the assessment of recombination events between two different loci. In addition, linkage analysis also depends on the availability of suitable genetic

markers.[69] These genetic markers should be highly polymorphic and distributed over the entire genome to allow a whole genome search for disease loci.

1.4.2.1. Homozygosity mapping

Homozygosity mapping is a technique used to locate stretches of homozygosity in the genome, mostly for recessive diseases, that are shared by the affected individuals in a family. Next, these regions are searched for the obvious disease-causing genes, which are sequenced to identify the possible disease causing mutations.

Homozygosity mapping can be performed using several hundred microsatellite markers spread over the entire genome, but more recently, single nucleotide polymorphism (SNP)-microarrays are employed that contain between 10,000 and 1,000,000 SNPs equally spread over the genome.[70]

Genotyping microsatellite markers

Simple sequence repeats known as microsatellite markers are highly polymorphic DNA sequences composed of di-, tri- and tetra-nucleotide repeats located on different chromosomes. Because of the genome-wide distribution of these microsatellite markers they are commonly used to map different disease causing genes across the human genome. In this method of homozygosity mapping microsatellite markers are used to genotype the individuals to find the homozygous stretches of DNA on different chromosomes, known as haplotype blocks, which are shared by all the affected individuals of a family. These shared regions are further investigated to locate the candidate genes, which harbour the disease causing mutations.

Significance of linkage is tested by calculating logarithm of the odds (LOD) score, using genotyped haplotype blocks in affected as well as healthy individuals.[71] Different linkage-analysis softwares are used to calculate LOD score, where a LOD score of at least 3.3 in a genome-wide analysis is considered to be significant .[69]

Genotyping single nucleotide polymorphisms

Normal humans carry 23 pairs of chromosomes, one pair of which is inherited from each parent.[72] These chromosomes together carry approximately 25,000 genes that encode different proteins required for life. The genomic sequences of two unrelated individuals are ~99.5% identical, while the remaining 0.5% is different due to naturally occurring structural

variations, among which are the copy number variants (CNVs; insertions, deletions), inversions, and single nucleotide polymorphisms (SNPs). The latter in most cases represent natural variations that do not render any functional effects. Levy *et al*., identified nearly 3.2 million SNPs scattered throughout the genome of an individual,[72] which were present in the coding as well as non-coding DNA sequences. In the protein coding regions of the genome, SNPs can either be synonymous (i.e. there is no change in amino acid) or nonsynonymous (the amino acid is changed). Ng *et al*., identified 10,400 nonsynonymous SNPs in a human exome, of which only ~1500 were predicted to (slightly) affect protein function.[73] Because of the distribution of the SNPs throughout the genome they can potentially be a useful tool in the determination of homozygous regions in linkage analysis.

Any single defect or change in the genetic information stored on the chromosomes in the form of genes may result in the expression of a disease condition. In the case of autosomal recessive disorders, affected individuals inherit mutant alleles from both parents (i.e. they carry two different mutations and are compound heterozygous, or carry two identical mutations and are homozygous for this variant), whereas when a single allele is responsible for the manifestation of the abnormal phenotype it is classified as an autosomal dominant or X-linked recessive disease.

High-density SNP microarray analysis is a recently developed technique used for homozygosity mapping.[74] This technique allows the detection of smaller regions of homozygosity as compared to microsatellite markers because of the higher density of the SNPs compared to the microsatellite markers.

1.4.3. Exome sequencing

Exome sequencing employing next generation sequencing (NGS) platforms is the most recent technique used to identify novel disease associated genes. This technique is now becoming popular and is widely used in diagnostics as well as in research because of its robustness, high throughput, cost effectiveness and high turnover rate.[75,76] Exome sequencing can be employed to identify the genetic cause of rare as well as commonly inherited genetic disorders. In this technique exonic sequences of all genes are first enriched and then sequenced. Presently, there are a number of different NGS platforms, which are being used for enrichment and sequencing purposes.[77,78] A brief description of exome sequencing using the SOLiD™ (sequencing by oligonucleotide ligation and detection) platform in combination with Agilent enrichment kits is given here. Enrichment is carried out by shearing the DNA sample to

produce smaller fragments of 150-200 bps. Ends of these fragments are repaired to produce blunt ends with 5' phosphorylation, adenine overhangs are then added to the blunt-ended DNA fragments with the help of the Klenow fragment of DNA polymerase. Indexing-specific adaptors are ligated to these fragments, which are then PCR amplified followed by hybridization to the enrichment probe library, which contains biotinylated ribonucleic acid (RNA) probes. Hybridized fragments are selected by streptavidin coated magnetic beads, RNA probes are then digested and enriched DNA is eluted to be used in further downstream process. For DNA sequencing in a SOLiD™ sequencer from Life Technologies, a ligation mediated di-base detection system along with a set of five universal sequencing primers is used.[77,79] The sequence read length generated by the SOLiD™ sequencer was initially 35 bp, which has now been improved to 50 bps. In the first step universal sequencing primers are annealed that bind to adapter sequences, followed by annealing and ligation of the 8-mer probe, which contains two colour-encoded bases at the 3' end and a fluorescent label at the 3' end. Fluorescence detection follows cleavage and removal of three bases at the 3' end of the 8-mer probe along with the fluorescent label. This cycle is repeated ten times followed by denaturation and removal of the first synthesized oligonucleotides. Each universal primer is then offset by a base followed by probe annealing, ligation, fluorescence detection and cleavage of fluorescent label to detect the remaining bases. The DNA template sequence is retrieved by decoding the sequence of detected colours. Sequencing reads are then aligned against a reference assembly to find the variants. The exome sequencing data are filtered to narrow down the number of variants to ease the identification of the causative mutation.

This technique can be highly advantageous in novel gene discovery, when used in combination with homozygosity mapping data, by selecting exome sequencing present in the identified linkage regions or, in case of recessively inherited disorders, in the homozygous regions. In addition, this technique is also suited for the identification of compound heterozygous mutations.

1.5. Treatment and prevention of inherited eye diseases

With the advent of modern techniques in molecular biology, our knowledge base has increased enormously in the field of disease genetics. A large number of studies are now being conducted to correct the non-functional or aberrant copy of the causative genes using different techniques. Currently patients are benefitting from successful gene therapy experiments in the western world. In addition to these genetic corrections, some of the genetic

diseases like FA can be treated with oral supplementation. Another option for the patients with end stage disease is retinal transplantation. Electronic devices are also being developed for the treatment of inherited retinal disorders. However, genetic counseling is the foremost option available to the patients and affected families for disease prevention in the next generations.

1.5.1. Gene therapy

Being an easily accessible and highly compartmentalized organ,[80] the eye is most suitable for gene-augmentation therapy for the treatment of inherited ocular disorders like Leber congenital amaurosis (LCA), RP, cone dystrophy (CD), cone-rod dystrophy (CRD), Usher syndrome, and BBS. Gene therapy involves delivery of a functional copy of the defective gene into the target tissue through an appropriate vector. To date, adeno-associated viruses (AAVs) are the most commonly used vector for retinal gene delivery[80] because AAV not only has the ability to transduce RPE as well as rod and cone photoreceptors,[81,82] but also remains extranuclear (episomal) thereby posing no threat of insertion in critical human genes, such as tumor suppressor genes.

After successful trials of AAV-mediated gene therapy of the *RPE65* gene in *Rpe65*$^{-/-}$dogs[83] LCA patients were selected for phase 1 clinical trials.[84-86] This therapy was found to be safe as no toxic effect or immune responses were noted, and the treated persons showed moderate visual improvements. AAV-mediated *RPE65* gene augmentation therapy has provided hope that similar future trials can be conducted for other disease causing genes.[87] At the moment, gene augmentation trials are also underway for Stargardt disease (*ABCA4*), choroideremia (*CHM*), LCA and arRP (*RPE65*), Usher syndrome type 1B (USH1B; *MYO7A*) and retinal disease patients with mer tyrosine kinase (*MERTK*) mutations (http://clinicaltrials.gov/ct2/home).

In addition to the above mentioned genes for which gene therapy trials are in progress in humans after success in animal models, several other genes are in line to be tested for gene therapy in animal models.[30]

Another option to restore visual function is to orally administer 9-*cis* retinal, as this oral supplementation has shown improvement in visual function in *Rpe65*$^{-/-}$ mice[88] and should be considered as a potential therapeutic option for patients.

1.5.2. Food supplementation

According to Jacobson and Cideciyan[2] slowing down the degeneration of photoreceptors by using different food supplements and other pharmacological agents is another useful strategy for the treatment of inherited eye disorders. Use of molecules that inhibit the apoptotic pathway significantly reduces the accumulation of toxic compounds in the retinal cells, as a result of which photoreceptor degeneration slows down. Vitamin A supplementation is also an option helpful in reducing photoreceptor damage.[89]

1.5.3. Retina transplants and electronic devices

In patients with advanced retinal degenerations in which the photoreceptors or RPE are completely lost, there is an option of retinal transplants from donors.[2] In a recent study, patient-specific induced pluripotent stem cells were shown to differentiate into RPE-like tissue and when transferred to the subretinal space resulted in the restoration of visual function in mice.[90] Therefore, subretinal injection of genetically engineered regenerated non-retinal cells can also be potentially used to treat the patients.[2]

Although gene therapy and food supplementation are good preventive measures to restore vision, when the photoreceptors are completely degenerated these techniques cannot make any significant improvement and electronic retinal implants remain the only option left for the patients.[91] These implants are positioned subretinally or epiretinally and stimulate the inner retinal neuronal cells by converting light signals into electric impulses.[91] Recently an electronic device with 16 electrodes was successfully implanted in patients with encouraging results and has prompted the development of devices with a higher number of electrodes to improve vision to a level to allow the patients to perform daily activities.[92] In the future it is hoped that the electronic retinal implants will become a robust therapeutic alternative, especially for individuals with severe eye diseases.

1.5.4. Genetic counseling

In molecular genetic research, individuals and families with inherited disorders are enrolled to determine the molecular basis of the diseases by identifying the causative mutations in their genomes. After determination of the causative mutation the results can be conveyed to the respective families, who can then be advised to take acceptable measures to prevent further disease occurrence in the family. Additionally, when there is a possibility of therapeutic intervention, patients can be referred to a consultant for suitable medication and treatment.

This whole process of disseminating information and suggestions to the respective families is known as genetic counseling.

Genetic counseling is very important in managing the disease in affected individuals and for preventing the further spread of the disease in the family. Genetic counseling is basically a communication process or psychotherapeutic intervention in individuals based on the results of genetic testing or screening outcomes.[93]

Depending upon the inheritance pattern of the disease, carriers of genetic mutations should ideally select their spouses carefully so as to minimize the chance of bearing affected children. Recessively inherited retinal diseases mostly have severe consequences whereas dominantly inherited disorders are mostly milder in nature. Inherited diseases that result from mutations in genes that reside on the autosomes affect males and females equally, but genes on the X-chromosome behave in a different way. Males with an X-linked disorder have a more severe phenotype as compared to the females because of the presence of a single X-chromosome in males.[34] In addition, X-linked inheritance can have a dominant or recessive mode of inheritance. Affected males usually have healthy parents since their mothers remain asymptomatic carriers of the genetic defect.[69]

The success of genetic counseling remains dependent on the correct diagnosis and appropriate genetic testing. Counseling can be very accurate for genes that have established genotype-phenotype correlations but it becomes challenging when there is a greater genetic and phenotypic heterogeneity. Thus, the medical advisor or geneticist who deals with families and individuals regarding their genetic testing must be a trained genetic counselor. It must be kept in mind that genetic counseling of families with *de novo* mutations is a challenging task for genetic counselors because of the absence of a family history and healthy parents not carrying the disease causing mutations.

In Pakistan, genetic counseling services are rare and because of consanguineous marriages the occurrence of genetic diseases cannot be properly managed, therefore, the number of affected individuals is increasing every day. In addition, there is no established prenatal diagnosis service for families with severely affected individuals. Establishment of functional genetic counseling services is urgently needed to manage genetic disorders in Pakistan, in order to convince and educate the affected families about the utility of the services.

1.6. Molecular genetics of eye diseases

In the last couple of decades significant volume of scientific work has been conducted in the field of ophthalmogenetics. As a result of combined efforts of life scientists worldwide, scientific knowledge has grown rapidly and developments in the field of molecular biology have enabled molecular geneticists to identify the mutated genes, which are the underlying cause of different genetic disorders. To date more than 200 genes and loci have been identified to be associated with retinal eye diseases (https://sph.uth.tmc.edu/retnet/).

1.6.1. Genetics of eye diseases in Pakistan

In Pakistan, because of the custom of consanguineous marriages and lack of knowledge about inherited disorders, such diseases are becoming more frequent. A number of research groups are now working in Pakistan to explore the genetics of inherited eye disorders. The most common inherited eye disorder in Pakistan is RP;[94-101] other less commonly found diseases include LCA,[102-107] fundus albipunctatus,[67,108] Oguchi disease[109,110] and achromatopsia.[111-113] In addition to these non-syndromic forms of retinal diseases, some syndromic forms are also found including Usher syndrome,[114-117] BBS[118-123] and Senior-Loken syndrome.[124]

An overview of the genes, that have thus far been found to be mutated in non-syndromic and syndromic retinal diseases in the Pakistani population, is given in Table 1.

Table 1.1 Overview of genes associated with non-syndromic and syndromic forms of retinal diseases in Pakistan

Gene	Disease	Function	Reference
ABCA4	arRP	Visual cycle	den Hollander, *et al*.,[125] Sun, *et al*.,[126] Sun, *et al*.[127]
AHI1	JBTS	Unknown	Louie, *et al*.[128]
AIPL1	EORP	Intracellular transport	van der Spuy, *et al*.,[129] Ramamurthy, *et al*.,[130] Akey, *et al*.[131]
ARL13B	JBTS	Ciliogenesis	Larkins, *et al*.[132]
BBS2	BBS	Intracellular transport	Nishimura, *et al*.[133]
BBS3/ARL6	BBS	Intracellular transport	Pretorius, *et al*.,[134] Pasqualato, *et al*.[135]
BBS4	BBS	Intracellular transport	Kim, *et al*.[136]
BBS5	BBS	Ciliogenesis	Li, *et al*.[137]
BBS8/TTC8	BBS	Ciliogenesis	Tadenev, *et al*.[138]
BBS10	BBS	BBSome assembly	Seo, *et al*.[139]

BBS12	BBS	BBSome assembly	Seo, *et al*.[139]
BEST1	adRP	Ion exchange	Burgess, *et al*.,[140] Marmorstein, *et al*.[141]
CC2D2A	JBTS	Ciliogenesis	Bachmann-Gagescu, *et al*.[142]
CDH23	USH1	Organization of stereocilia	Holme, *et al*.[143]
CEP290 (NPHP6)	JBTS	Transport across CC	Sayer, *et al*.[144]
CERKL	arRP	Unknown	Tuson, *et al*.[145]
CLRN1	arRP	Unknown	Khan, *et al*.[101]
CNGA1	arRP	Phototransduction cascade	Dhallan, *et al*.[146]
CNGA3	arCRD (ACHM)	Phototransduction cascade	Kohl, *et al*.[147]
CNGB1	arRP	Phototransduction cascade	Korschen, *et al*.[148]
CNGB3	arCRD (ACHM)	Phototransduction cascade	Nishiguchi, *et al*.[149]
CRB1	arLCA	Photoreceptor morphogenesis	Pellikka, *et al*.[150]
EYS	arRP	Integrity of photoreceptors	Collin, *et al*.,[151] Abd El-Aziz, *et al*[152]
GNAT1	arCSNB	Phototransduction cascade	Dryja, *et al*.[153]
GRK1	arCSNB	Phototransduction cascade	Khani, *et al*.[154]
IMPG2	arRP	Interphotoreceptor matrix organization	Bandah-Rozenfeld, *et al*.[155]
IQCB1 (NPHP5)	SLSN	Ciliogenesis	Otto, *et al*.[156]
LCA5	arLCA	Transport across CC	den Hollander, *et al*.[105]
MERTK	arRP	Rod outer segment phagocytosis	Vollrath, *et al*.[157]
NMNAT1	arLCA	Biosynthesis of NAD	Koenekoop, *et al*.[158]
NPHP4	SLSN	Unknown	Mollet, *et al*.[159]
PCDH15	USH1	Retinal and cochlear maintenance	Ahmed, *et al*.,[116] Alagramam, *et al*.[160]
PDE6A	arRP	Phototransduction cascade	Huang, *et al*.[161]
PDE6B	arRP	Phototransduction cascade	McLaughlin, *et al*.[162]
PROM1	arRP	Gene regulation	Yang, *et al*.[163]
RDH12	arLCA/EORD	Visual cycle	Haeseleer, *et al*.[164]
RDH5	arCSNB (FA)	Visual cycle	Yamamoto, *et al*.[45]
RHO	adRP	Phototransduction cascade	Nathans, *et al*.[165]
RLBP1	arCSNB (FA)	Visual cycle	Sparkes, *et al*.[166]
RP1	arRP	Rod outer segment morphogenesis	Liu, *et al*.,[167] Liu, *et al*.[168]
RPE65	arLCA	Visual cycle	Xue, *et al*.,[169] Moiseyev, *et al*.[170]
RPGR	xlRP	Intracellular transport	Linari, *et al*.[171]
RPGRIP1	arCRD	Transport across CC	Castagnet, *et al*.[172]
SAG	arCSNB (Oguchi)	Phototransduction cascade	Yamaki, *et al*.[173]
SEMA4A	arRP/arCRD	Cell to cell communication	Rice, *et al*.[174]
SLC24A1	arCSNB	Ion exchange	Riazuddin, *et al*.[175]
SPATA7	arLCA/arRD	Unknown	Wang, *et al*.[176]
TCTN2	JBTS	Ciliogenesis	Sang, *et al*.[177]
TMEM67	MKS	Ciliogenesis	Smith, *et al*.[178]

TTC8	arRP	Ciliogenesis	Riazuddin, *et al*.[179]
TULP1	arRP	Transport across CC	Hagstrom, *et al*.[180]
USH1G (SANS)	USH1	Retinal and cochlear maintenance	Weil, *et al*.[181]

Legend: ACHM, achromatopsia; ad, autosomal dominant; ar, autosomal recessive; BBS, Bardet-Biedl syndrome; CSNB, congenital stationary night blindness; CRD, cone-rod dystrophy; EORD: early onset retinal dystrophy; EORP, early onset arRP; FA, fundus albipunctatus; JBTS, Joubert syndrome; LCA, Leber congenital amaurosis; MKS, Meckel syndrome; NAD, nicotinamide adenine dinucleotide; CC, connecting cilium; RD, retinal dystrophy; RP, retinitis pigmentosa; SLSN, Senior Loken syndrome; USH1, Usher syndrome type 1; xlRP, X-linked RP

Populations with a higher rate of consanguineous marriages are at a greater risk of having genetic disorders, and therefore are considered as a useful source for studying the molecular basis of the diseases. According to an estimate more than 60% marriages in Pakistan are consanguineous, of which nearly 80% are estimated to be among first cousins.[182,183] Because of this high rate of consanguinity, there are higher chances of inheriting a disease causing variant in the homozygous state by offspring of heterozygous carrier parents, which can lead to disease.[183] As most of the families included in this thesis were consanguineous, homozygosity mapping was employed to identify the causative variants.

1.7. Structure of this thesis

The aim of the work presented in this thesis was to identify genetic causes of inherited eye diseases in the Pakistani population. This study mainly focuses on the genetics of RP, FA and BBS. Homozygosity mapping combined with Sanger sequencing was mainly used to identify the different disease causing mutations in the affected families. In addition, exome sequencing was employed to find the underlying genetic cause of the disease in two BBS families and one RP family.

In the **second** chapter, two large consanguineous families with *TULP1* mutations are discussed. In addition, an overview of all the *TULP1* mutations identified so far has been given. The frequency of *TULP1* mutations in Pakistani patients has also been calculated.

The **third** chapter describes the genetics of FA in two Pakistani families. This is a rare genetic disease for which mutations have mostly been reported in *RDH5*. Although two other genes, *RLBP1* and *RPE65,* are also known to be associated with FA, their occurrence is very rare. The possibility of FA treatment is also discussed.

The **fourth** chapter consists of a description of a novel splice site mutation and a recurrent missense mutation identified in the *BBS1* gene that were identified through exome sequencing in two families segregating BBS. It also highlights the use of exome sequencing in identifying genetic mutations.

In the **fifth** chapter, mutations in *ABCA4*, *AIPL1*, *CERKL* and *PDE6A* are described to be the underlying genetic causes of retinal dystrophy in four consanguineous families. A novel mutation was identified in *PDE6A* whereas previously identified mutations were present in *ABCA4*, *AIPL1* and *CERKL*.

In the **sixth** chapter, the genetics of syndromic and non-syndromic retinal dystrophies in Pakistan was reviewed along with reporting two novel mutations in *CNGB1* and *CNGA1*. In addition, several novel loci for RP were identified.

In the **seventh** chapter, a novel candidate gene for severe RP, *DHX38,* encoding a protein involved in RNA splicing (PRP16), was reported.

In the **eighth** chapter, the general discussion, the broader impact of the current research was provided. The power of homozygosity mapping, genetic heterogeneity, phenotypic variability and the possibility of modifier alleles were discussed. In addition, genotype-phenotype correlations, possibility of therapeutic intervention; linking clinician and geneticist; benefits to the patients and families; and future research directions were addressed.

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Chapter 2

Identification of known and novel mutations in *TULP1* in Pakistani families with early-onset retinitis pigmentosa

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ABSTRACT

Purpose: To identify the genetic defects underlying retinitis pigmentosa (RP) in Pakistani families.

Methods: Genome-wide high-density single-nucleotide-polymorphism microarray analysis was performed using the DNA of nine affected individuals from two large families with multiple consanguineous marriages. Data were analyzed to identify homozygous regions that are shared by affected sibs in each family. Sanger sequencing was performed for genes previously implicated in autosomal recessive RP and allied retinal dystrophies that resided in the identified homozygous regions. Probands from both families underwent fundus examination and electroretinogram measurements.

Results: The tubby-like protein 1 gene (*TULP1*) was present in the largest homozygous region in both families. Sequence analysis identified a previously reported mutation (c.1138A>G; p.(Thr380Ala)) in one family and a novel pathogenic variant (c.1445G>A; p.(Arg482Gln)) in the other family. Both variants were found to be present in a homozygous state in all affected individuals, were heterozygous present in the unaffected parents, and heterozygous present or absent in normal individuals. Affected individuals of both families showed an early-onset form of RP.

Conclusions: Homozygosity mapping, combined with candidate-gene analysis, successfully identified genetic defects in *TULP1* in two large Pakistani families with early-onset retinitis pigmentosa.

2.1. Introduction

The major cause of inherited blindness in humans is retinitis pigmentosa (RP; OMIM 268000). The clinical symptoms of RP are the loss of night vision in the early phase of disease, later followed by peripheral vision loss, tunnel vision, and sometimes complete blindness.[1] Progression of the disease is mainly caused by the gradual loss of rod photoreceptor cells, which are mostly responsible for vision under low light conditions, and the subsequent loss of cone photoreceptor cells, which are involved in color vision under bright light conditions. The clinical diagnosis is based on fundus examination and electrophysiological analysis of rod and cone photoreceptor-cell function by measuring the scotopic and photopic responses, respectively, using electroretinography (ERG). The disease's characteristics are the presence of pigmentary deposits (bone spicules) in the peripheral fundus, diminished or no ERG responses from rod and cone photoreceptor cells, and attenuation of the retinal blood vessels.[1]

The disease is highly genetically heterogeneous, since 55 different genes and three loci have been identified as being associated with nonsyndromic RP (RetNet, Nov. 8, 2011). All Mendelian forms of inheritance have been observed for RP. Digenic forms and rare mitochondrial inheritance have also been reported.[1-4]

Tubby-like protein 1 (TULP1; OMIM 602280) belongs to the tubby-like protein family. The *TULP1* gene is located on the short arm of chromosome 6.[5] *TULP1* is expressed specifically in the retina,[5,6] and the encoded protein is thought to be involved in protein trafficking, such as the transport of rhodopsin from the inner segment to the outer segment via the connecting cilium.[7]

The aim of the current study was to identify the underlying genetic causes of autosomal recessive RP (arRP) in Pakistani families by using genome-wide homozygosity mapping and Sanger sequencing of known retinal disease genes in the homozygous regions. We identified disease-causing mutations in *TULP1* in two large families.

2.2. Methods

2.2.1. Ethics committee/institutional review board approval

Before initiating this study, approval for this work was granted by the Ethics Committee/Institutional Review Board of the Shifa College of Medicine/Shifa International Hospital, Islamabad, Pakistan, and signed informed consent was obtained from all participating individuals in both families.

2.2.2. Ascertainment of families and clinical analysis

Two families from the Punjab province with individuals having night vision and daytime vision loss were included in the study. Their pedigrees were drawn (Figure 2.1A,B) using HaploPainter,[8] and venous blood samples were collected in acid citrate dextrose-containing vacutainers (Becton Dickinson, Franklin Lakes, NJ). Fundus examinations were done, and ERG[9] measurements were made for probands in both families.

2.2.3. Genotype analysis and homozygosity mapping

DNA was extracted by using a standard phenol chloroform extraction protocol[10] and stored at 4 °C. HumanOmniExpress (>700K) single nucleotide polymorphism (SNP) microarrays from Illumina Inc. (San Diego, CA) were employed to search for homozygous regions in six affected individuals (Figure 2.1A) in family A and three affected individuals (Figure 2.1B) in family B. Genotype data were analyzed with Homozygosity Mapper,[11] an online tool for homozygosity mapping using SNP genotyping data. Haplotype comparisons were also done for affected and normal individuals to identify the homozygous regions that were identical in all the affected individuals in each family.

2.2.4. Sequence analysis and mutation detection

Prior to Sanger sequencing, the 15 protein-coding exons of *TULP1* (NM_003322.3) and their flanking intronic sequences were amplified by PCR using standard conditions and reagents. PCR primers were designed with the online primer-designing tool Primer3[12] (available on request). Amplified PCR products were electrophoresed in 2% agarose gels containing ethidium bromide, and DNA bands were visualized on an ultraviolet transilluminator. PCR products were purified on PCR clean-up purification plates (NucleoFast™ 96 PCR, Cat. No. 743100.10; MACHEREY-NAGEL, Düren, Germany), according to the manufacturer's

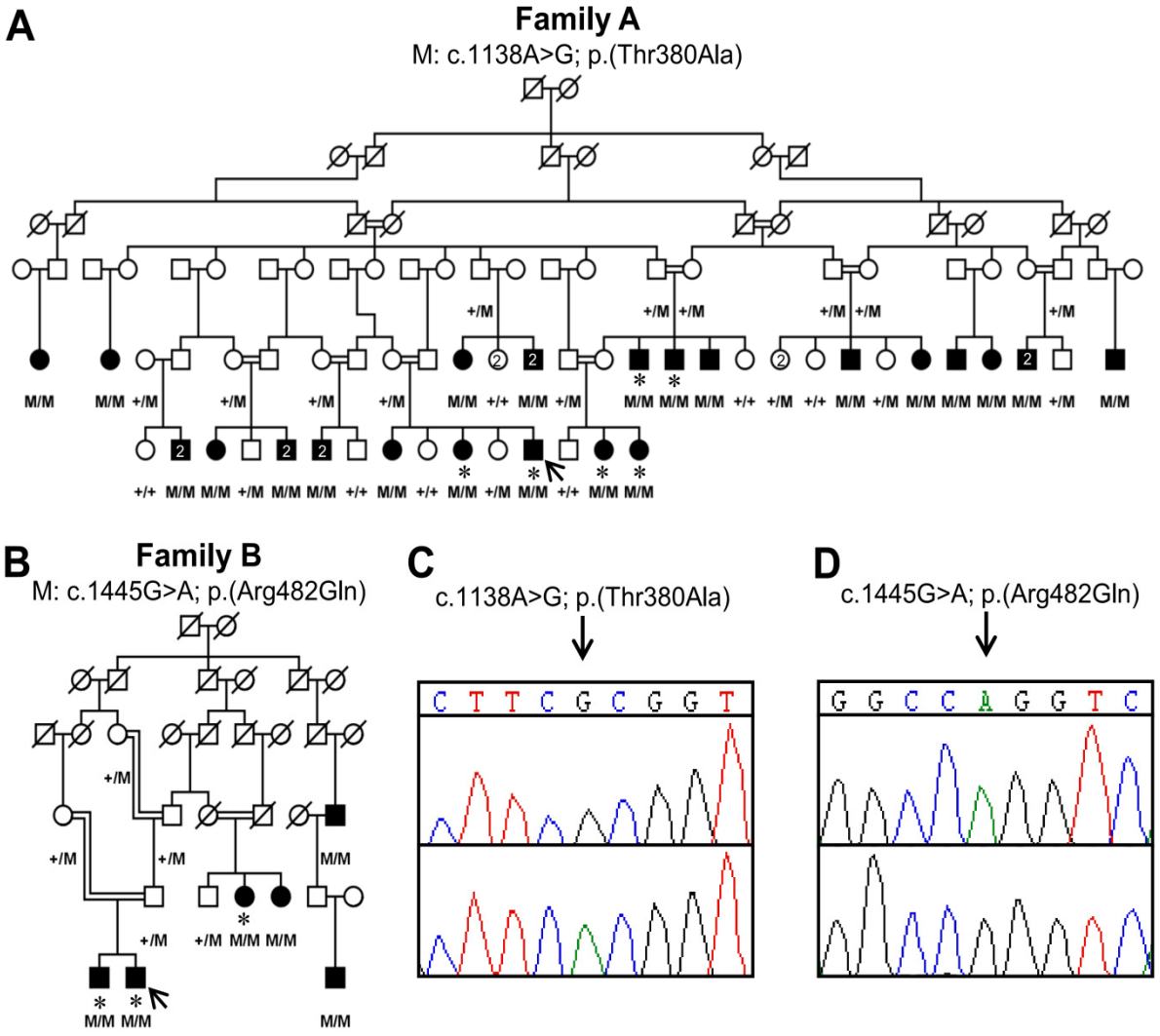

Figure 2.1 Pedigrees and *TULP1* genotyping results for families A and B. A: This is the pedigree of family A in which the presence of the c.1138A>G variant (M) was found in a homozygous state in all affected individuals. As expected for causal autosomal recessive variants, unaffected parents are heterozygous, and normal individuals carry one or no mutant allele. **B:** This is the pedigree of family B in which the presence of the c.1445G>A variant (M) was shown in a homozygous state in the 6 affected individuals, and in heterozygous state in an unaffected sibling, parents and grandparents of affected persons. **C:** This is the sequence electropherogram showing the nucleotide change from adenine to guanine in the proband of family A (upper panel), and sequence electropherogram of a control individual showing the wild-type adenine (lower panel). **D:** This is the sequence electropherogram of the proband carrying the mutant adenine in family B (upper panel); and the sequence electropherogram of a control individual with the normal guanine (lower panel). Probands are indicated with arrows; asterisks indicate the individuals that were tested using HumanOmniExpress (>700K) SNP microarrays. M/M, homozygous c.1138A>G (in family A) or homozygous c.1445G>A (in family B); +/M, heterozygous mutations present; +/+, two wild-type alleles present.

protocol. Purified PCR products were subsequently used for Sanger sequencing in an automated DNA sequencer (BigDye Terminator, version 3, on a 3730 DNA analyzer; Applied Biosystems, Foster City, CA).

Sequencing results were analyzed by using Vector NTI Advance™ 2011 software from Life Technologies/Invitrogen (Bleiswijk, Netherlands), by assembling the sequenced contigs and then visualizing the aligned sequences of the exons.

2.2.5. Pathogenicity assessment of identified variants

Identified missense variants were assessed for possible causality by using Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html) analysis and Polymorphism Phenotyping v-2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/).

2.2.6. Restriction fragment length polymorphism analysis

Restriction fragment length polymorphism analysis was performed to detect the presence or absence, of the identified mutations, in 100 ethnically matched control individuals. For the mutation identified in family A, restriction enzyme HpyCH4III was used, whereas in family B, restriction enzyme MspI was used. In both families, restriction enzyme recognition sites were abolished in the mutant sequences. Purified PCR products were used for restriction enzyme digestion, according to the manufacturer's protocol (New England BioLabs, Ipswich, MA).

2.2.7. Evolutionary conservation of amino acids

To check the evolutionary conservation of the mutated amino acids, the TULP1 orthologous protein sequences of the following species were aligned with the Vector NTI Advance™ 2011 software: humans (*Homo sapiens*, ENSP00000229771); chimpanzees (*Pan troglodytes*, ENSPTRP00000007764); mice (*Mus musculus*, ENSMUSP00000049070); dogs (*Canis familiaris*, ENSCAFP00000001922); chickens (*Gallus gallus*, ENSGALP00000009613); frogs (*Xenopus tropicalis*, ENSXETP00000000899); tetraodons (*Tetraodon nigroviridis*, ENSTNIP00000004001); fruitflies (*Drosophila melanogaster*, FBpp0088961); honeybees (*Apis mellifera*, GB19892-PA); roundworms (*Caenorhabditis elegans*, F10B5.4), blood flukes (*Schistosoma mansoni*, Smp_058730__mRNA); and Arabidopsis (*Arabidopsis thaliana*, AT1G76900.1).

2.2.8. Three-dimensional structure prediction

Project HOPE[13] (http://www.cmbi.ru.nl/hope/home) was used to predict the possible structural changes in the mutant TULP1 proteins identified in our study using a normal human TULP1 structure (PDB-file 3C5N).

2.3. Results

In both families, the average age of disease onset was in the first decade of life. Ophthalmic examination of affected individuals from both families revealed the presence of attenuated retinal vessels and the optic disc to have a waxy, pale appearance (Figure 2.2). A yellow perifoveal annular ring, a characteristic feature of individuals carrying *TULP1* mutations, was also clearly visible in family A (Figure 2.2A,B).

In family B, the perifoveal ring was in the process of development (Figure 2.2C,D). Upon ERG, scotopic and photopic electrophysiological responses of rod and cone photoreceptors, respectively, were diminished in affected members of both families (Table 2.1). Neither nystagmus nor eye poking were present in either family.

Genome-wide SNP microarray data of six affected individuals of family A were analyzed with the help of Homozygosity Mapper, which revealed a single homozygous region (Figure 2.3A) of 3.9 Mb (from 32.9 Mb to 36.8 Mb; hg19) on chromosome 6, flanked by SNPs rs3132131 and rs236411. This homozygous region harbored *TULP1*, a gene known to be mutated in patients with Leber congenital amaurosis (LCA) and arRP. The sequence analysis identified a previously reported mutation, c.1138A>G (p.Thr380Ala)[14,15] in this family (Figure 2.1C).

Similarly, genome-wide SNP microarray data analysis of three affected individuals of family B, resulted in the identification of six homozygous regions (Figure 2.3B). After haplotype comparison, two regions, a 4.8 Mb region on chromosome 6 (from 33.8 Mb to 38.6 Mb, flanked by SNPs rs9296102 and rs7761629) and a 1.4 Mb region on chromosome 7 (from 132.3 Mb to 133.7 Mb, flanked by SNPs rs924368 and rs10249912), were found to be identical in all the affected individuals. *TULP1* resides in the homozygous region on chromosome 6, and the sequence analysis revealed a novel mutation, c.1445G>A (p.(Arg482Gln); Figure 2.1D). The homozygous chromosomal region on chromosome 7 did not contain a gene previously implicated in an inherited retinal disease such as arRP or LCA.

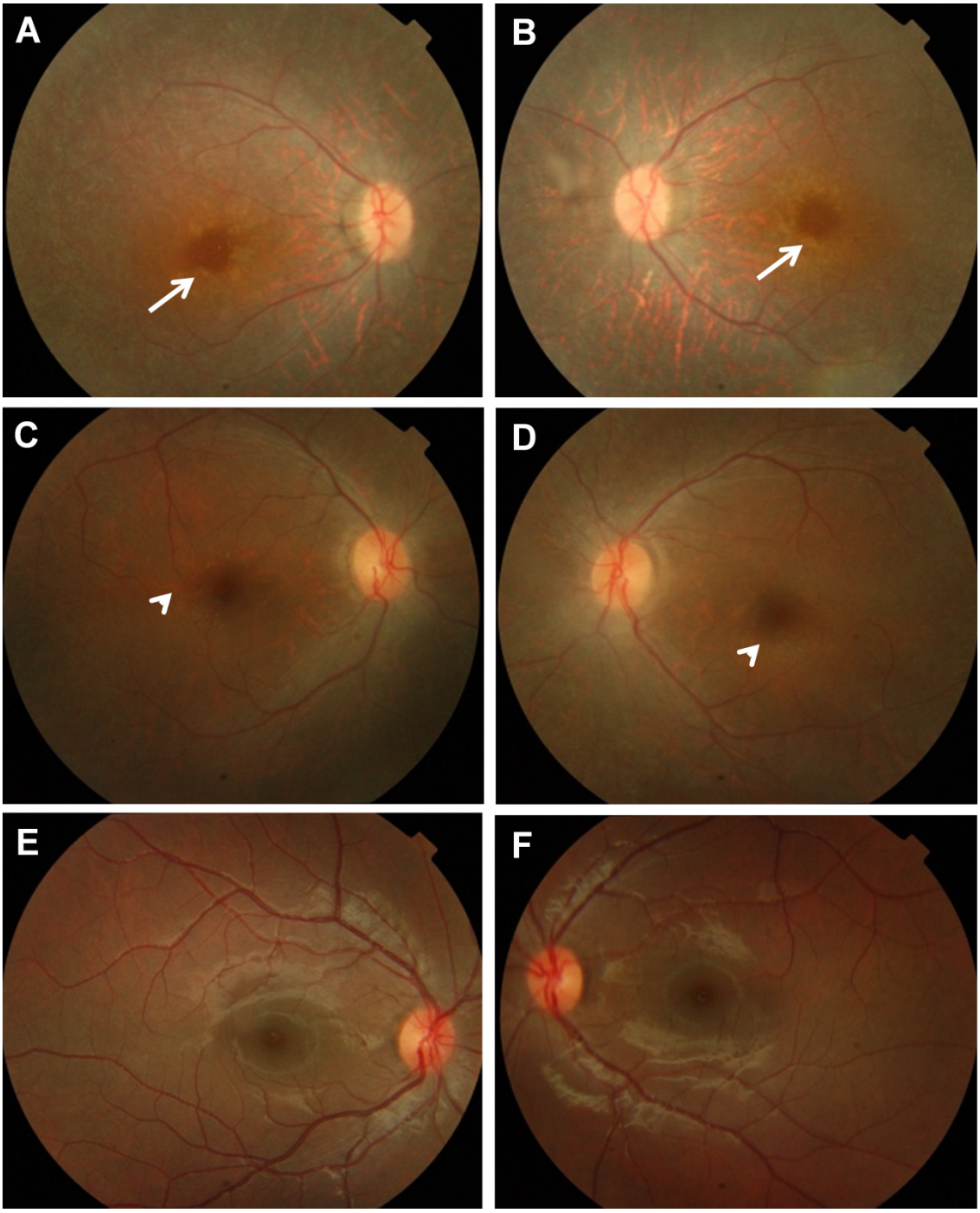

Figure 2.2 Fundus photographs of affected individuals from both families and of a normal individual. A, B: Right and left fundus, respectively, of the proband of family A (see arrow, Figure 2.1A), representative of the fundus appearance of all affected members of this family. Arrows indicate yellow perifoveal annular rings. **C, D:** Right and left fundus, respectively, of the proband of family B (see arrow, Figure 2.1B). Arrowheads point to the developing perifoveal annular rings. **E, F:** Right and left fundus, respectively, of a normal individual.

Table 2.1 Comparison of ERG responses of affected individuals of families A and B with a control individual.

Measured parameters using monopolar electrodes	Adaptation	Flash strength (cds/m^2)	Proband Family A	Proband Family B	Control	Normal values (Age=20 years)
Scotopic 25 dB b-wave amplitude (μV)	Dark	0.01	13.30	21.80	244.40	>185
Scotopic 0 dB b-wave amplitude (μV)	Dark	3.0	22.70	22.20	650.00	>419
Oscillatory potential amplitude (μV)	Dark	3.0	56.40	47.90	187.70	>110
Photopic 0 dB b-wave amplitude (μV)	Light	3.0	12.00	15.70	86.90	>102
Photopic 30 Hz flicker amplitude (μV)	Light	3.0	6.10	1.46	65.60	>70

Age of affected individuals from families A and B at the time of investigation was 20 years.

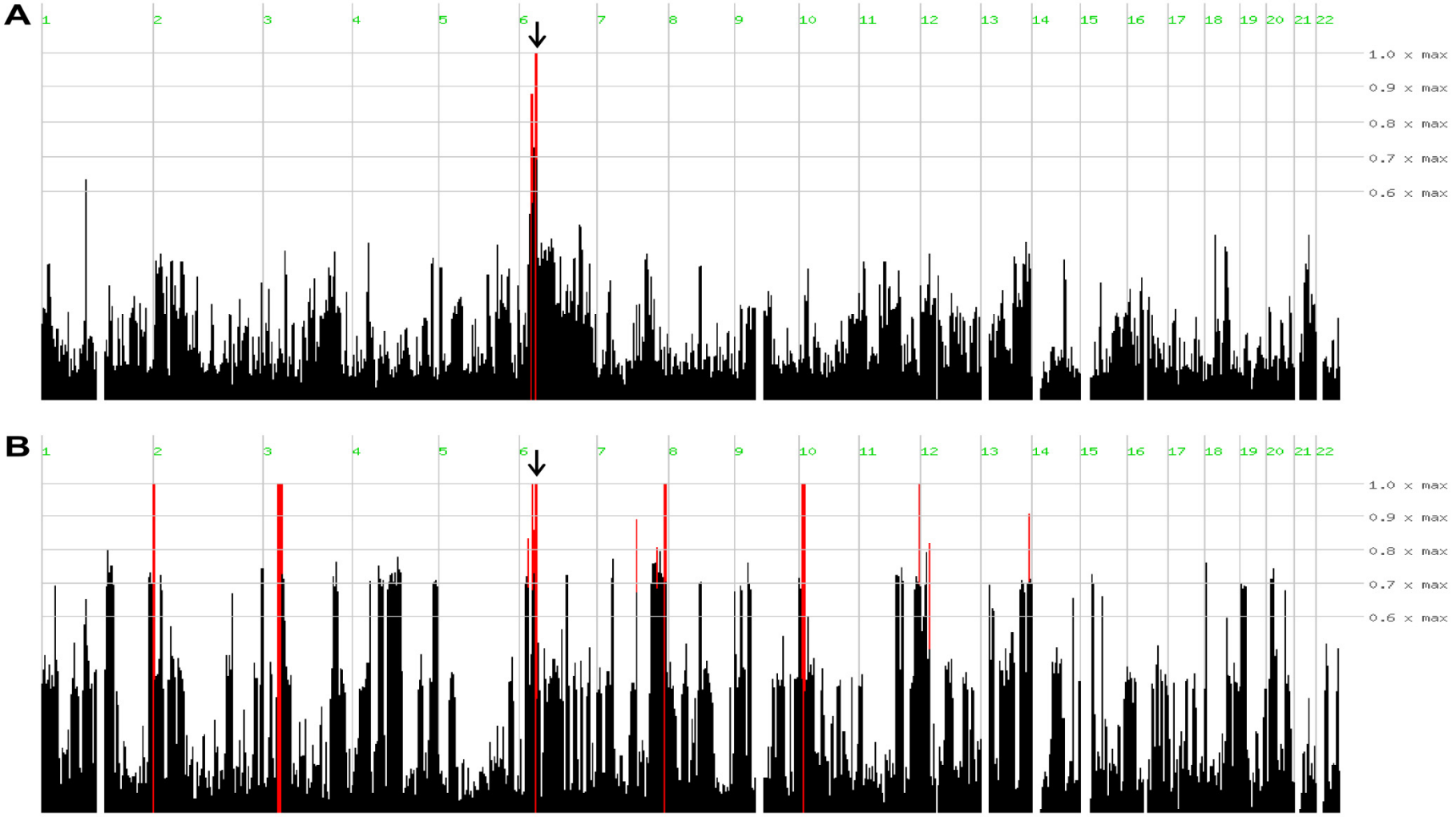

Figure 2.3 Homozygosity mapping results for families A and B. Homozygosity-mapper plots show the homozygous regions in the affected individuals in both families (red lines). Homozygous regions that show identical haplotypes for all affected individuals within a family are indicated by arrows. Panel A represents family A and panel B represents family B.

In families A and B, the variants c.1138A>G and c.1445G>A, respectively, were found to be present in a homozygous state in all affected individual, were heterozygous present in the unaffected parents, and absent or heterozygous present in normal individuals (Figure 2.1A,B). Both wild-type nucleotides were shown to be highly conserved, as evidenced by their phylogenetic P-value[16] scores of 2.87 (c.1138A) and 6.10 (c.1445G) for family A and B, respectively. In addition, the encoded amino acids, p.Thr380 and p.Arg482, located in the C-terminal tubby domain, are highly conserved among different vertebrate and invertebrate species, while in a plant (Arabidopsis), isoleucine is present instead of threonine (Figure 2.4). These amino acids are completely conserved among the tubby, TULP1, TULP2, and TULP3 proteins.[17] SIFT predicts that both TULP1 variants are "not tolerated" whereas PolyPhen-2 predicts that both are "probably damaging" with prediction scores of 0.827 and 1.000 for p.(Thr380Ala) and p.(Arg482Gln), respectively.

A three-dimensional-structure prediction analysis by project HOPE predicts that the p.(Thr380Ala) mutation, due to the smaller size of the alanine residue, causes an empty space in the protein and possible rearrangements of surrounding residues (Figure 2.5A,B). Any hydrogen bonds made by threonine will also be lost, because alanine is a hydrophobic residue. Very close to Thr380 is a predicted inositol triphosphate binding site that might also be influenced by local conformational changes. The p.(Arg482Gln) variant changes a positively charged amino acid (arginine) to a neutral residue (glutamine), which results in the loss of interactions with negatively charged residues in its vicinity (Figure 2.5C). In view of its location in the three-dimensional structure, these changes may result in a loss of external interactions. The p.(Thr380Ala) and p.(Arg482Gln) variants were not detected in 100 healthy ethnically matched control individuals.

2.4. Discussion

In the current study, we report on two large families with several consanguineous marriages and multiple individuals with early-onset RP that were found to carry *TULP1* mutations. Family A had 27 affected individuals who resided in the northern part of the Punjab province. This family had a strong custom of marrying inside the family. *TULP1* mutations were previously reported to cause LCA, a congenital form of severe vision impairment or blindness, and early-onset RP.[14,15,18-31] There is clinical and genetic overlap between (early-onset) arRP and LCA,[32,33] and often it is very difficult to differentiate between these two conditions. Mutations in Crumbs homolog 1 (*CRB1*), Lecithin retinol

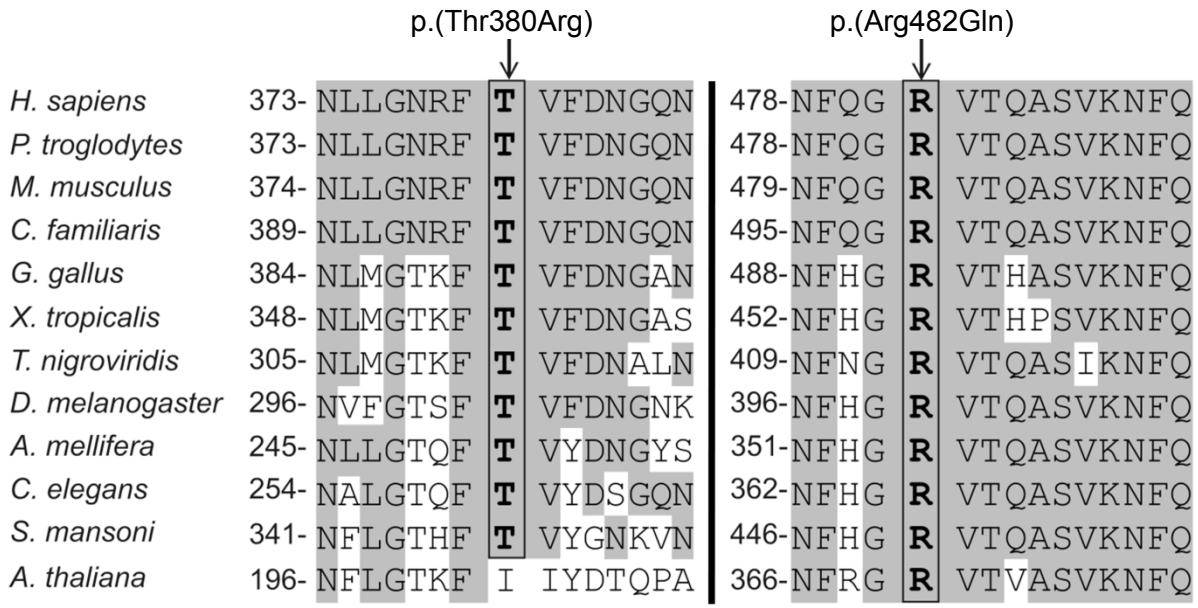

Figure 2.4 Conservation of mutated amino acids among different species. Amino acids identical to human tubby-like protein 1 (TULP1) amino acids are shaded in gray. The positions of the amino acids in the respective polypeptides are indicated.

acyltransferase (*LRAT*), Mer tyrosine kinase protooncogene (*MERTK*), Retinal pigment epithelium-specific protein, 65-KD (*RPE65*), Spermatogenesis-associated protein 7 (*SPATA7*), and *TULP1* have been identified in individuals with LCA and arRP.[18-21,34-47] If a severe form of rod–cone dystrophy is observed beyond early childhood, and when there are no features reminiscent of LCA, such as nystagmus, or eye poking, the phenotype is classified as early-onset RP.

TULP family proteins (TUB, TULP1, TULP2, and TULP3) have crucial roles in embryonic development in vertebrates and take part in the proper functioning of the central nervous system.[6,48] *TULP1* is expressed specifically in the rod and cone photoreceptor cells,[5,6] and is involved in the transport of rhodopsin.[7] In *Tulp1*$^{-/-}$ mice, photoreceptor degeneration precedes synaptic malfunction, and thus TULP1 may have a function in photoreceptor synapse development.[49] A mutation of the same residue (p.(Arg482Trp)) was earlier found in combination with p.(Leu504fs*141) in five affected individuals with severe early-onset RP.[50]

The structural analyses of the TULP1 C-terminal domains of the mutant proteins suggest that the missense mutations identified in our study might have resulted in the destabilization of the mutant proteins, or might have influenced the putative interactions of the tubby domain. Among different species of animals and plants, p.Arg482 is located in the signature sequence (F-[KRHQ]-G-R-V-[ST]-x-A-S-V-K-N-F-Q) of the Tubby family of proteins, and this

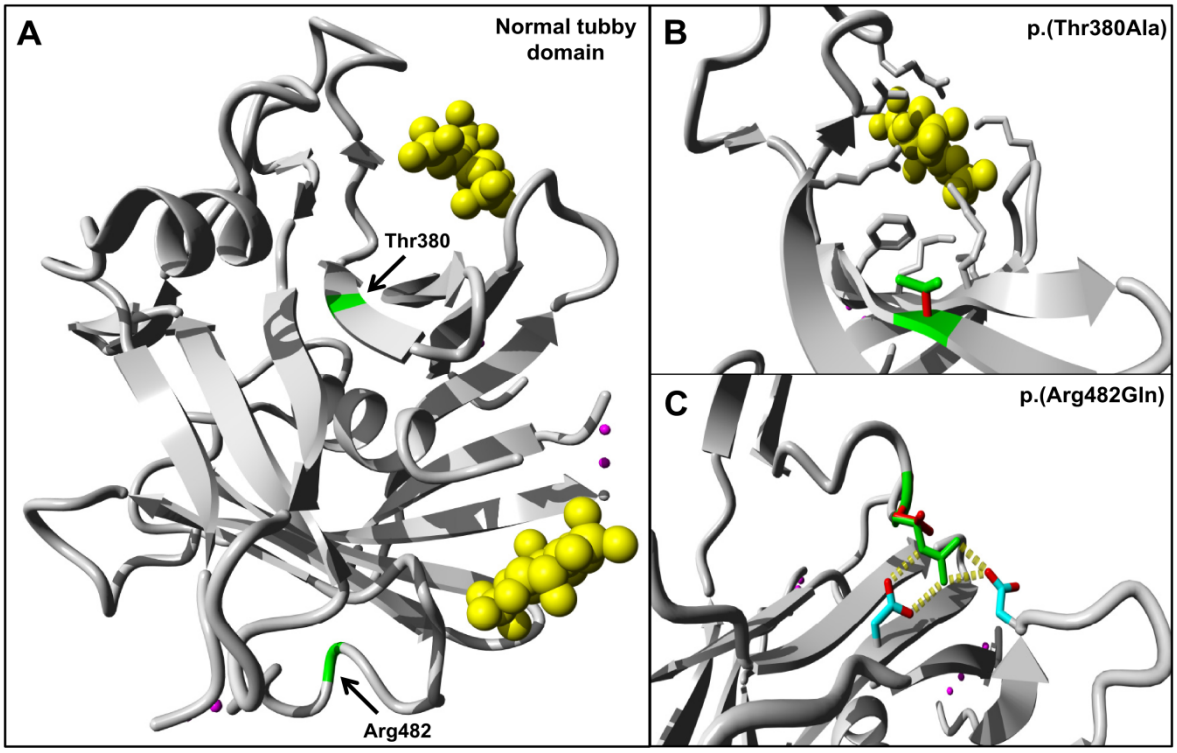

Figure 2.5 Three-dimensional domain architecture of the tubby domain of TULP1 wild-type and mutant proteins. A: Preferred predicted secondary structure of normal tubby-like protein 1 (TULP1) with Thr380 and Arg482 indicated in green. In yellow, the inositol triphosphate molecules that are predicted to bind the tubby domain of TULP1. **B:** Predicted structure of part of the p.(Thr380Ala) mutant protein in affected individuals of family A. The smaller size of the alanine residue may lead to rearrangements of surrounding residues and thereby affect putative inositol triphosphate binding. **C:** Part of the predicted structure of the p.(Arg482Gln) mutant protein found in affected individuals of family B. The p.(Arg482Gln) variant changes a positively charged amino acid (arginine) to a neutral residue (glutamine), which leads to loss of interactions with two negatively charged residues in its vicinity. Wild-type interactions are indicated with yellow blocks.

signature sequence contains 11 invariant amino acids that are highly conserved (Prosite).[5] Replacement of the wild-type residue with the mutant glutamine might affect the signature sequence; this might ultimately prevent the mutant TULP1 protein from functioning normally.

In family A, the presence of a typical yellow-colored perifoveal annular ring was also indicative of TULP1 involvement;[24] whereas in family B, the ring formation was incomplete (Figure 2.2C,D). The bone spicules absent from both families might still develop later in life. The age of both individuals who were clinically evaluated was 20 years.

The previously identified mutation p.(Thr380Ala) has only been reported in two unrelated Pakistani families.[14,15] Our findings concerning this mutation in yet another Pakistani family suggested that c.1138A>G might be a Pakistani founder mutation, although no link was

established between any of these families. One family belonged to a northern area of Pakistan[14] while the other belonged to the southern part of Punjab.[15] Our family belonged to the northern part of Punjab, which, however, does not exclude the possibility that this mutation represents a founder mutation in Pakistan.

Including our findings, 26 different *TULP1* mutations have been identified in 33 families (Table 2.2). *TULP1* mutations were found in 4.3% (10/231) of LCA families[14,18,24,25,27,29,30] and 2.4% (23/948) of arRP families.[15,19-24,26,28,30,31,50] *TULP1* mutations causing arRP or LCA include two nonsense, two frame-shift, and seven splice-site mutations; a six-base-pair duplication; and 14 missense mutations (Table 2.2). Splice-site and protein-truncating mutations are distributed throughout the gene (Figure 2.6A), whereas the missense mutations are only present in the C-terminal tubby domain of TULP1 (Figure 2.6B).

TULP1 mutations are a frequent cause of LCA and arRP, and therefore represent an attractive therapeutic target. Thus far, *TULP1* mutations have been found in a total of 136 individuals with LCA or arRP (Table 2.2). Through our studies, 33 additional patients with *TULP1* mutations might benefit from genetic counseling and future gene-augmentation therapy.

In conclusion, we were able to identify one novel (c.1445G>A; p.(Arg482Gln)) and one previously identified (c.1138A>G; p.(Thr380Ala)) disease-causing mutation in *TULP1* in Pakistani families with early-onset RP.

Table 2.2 *TULP1* mutations causing arRP or LCA.

Exon/intron	Mutations: Allele 1/Allele 2	Phenotype	No. of families	No. of cases	Reference
Intron 2, Exon 12	c.99+1G>A; p.(?)/ c.1204G>T; p.(Glu402*)	LCA	1/179	1/179	Hanein, *et al*.[18]
Intron 4, Exon 5, 10	c.350-2_350del; p.(?)/ c.901del; p.(Gln301Argfs*9)	arRP	1/49	2/49	Paloma, *et al*.[22]**
Intron2, Exon 14	c.99+1G>A; p.(?)/ c.1376T>A; p.(Ile459Lys)	arRP	1/536	1/536	Hagstrom, *et al*.[20]
Intron 7	c.718+2T>C; p.(?)/ c.718+2T>C; p.(?)	juvenile RP	2/86	2/86	den Hollander, *et al*.[24]
Exon 10	c.901C>T; p.(Gln301*)/ c.901C>T; p.(Gln301*)	LCA	5/37	42/117	Li, *et al*.[29]
Exons 10, 11	c.932G>A; p.(Arg311Gln)/ c.1025G>A; p.(Arg342Gln)	arRP	1/2	2/4	Hebrard, *et al*.[31]
Intron 10	c.999+5G>C; p.(?)/c.999+5G>C; p.(?)	juvenile RP	1/86	1/86	den Hollander, *et al*.[24]
Exon 11	c.1102G>T; p.(Gly368Trp)/ c.1102G>T; p.(Gly368Trp)	LCA	1/179	1/179	Hanein, *et al*.[18]
Exon 12	**c.1138A>G; p.(Thr380Ala)/ c.1138A>G; p.(Thr380Ala)**	LCA, **early onset arRP**	1/14, 1/5, **1/2**	3/64, 4/23, **27/33**	Mckibbin, *et al*.,[14] Iqbal, *et al*,[15] and **this study**
Exon 12	c.1145T>C; p.(Phe382Ser)/ c.1145T>C; p.(Phe382Ser)	arRP	1/59	2/59	Kondo, *et al*.[23]
Exon 12	c.1199G>A; p.(Arg400Gln)/ c.1199G>A; p.(Arg400Gln)	arRP	1/34	2/34	Singh, *et al*.[28]

Exon 12	c.1198C>T; p.(Arg400Trp)/ c.1198C>T; p.(Arg400Trp)	LCA	1/179	1/179	Hanein, *et al*.[18]
Exons 13, 14	c.1259G>C; p.(Arg420Pro)/ c.1471T>C; p.(Phe491Leu)	arRP	1/536	2/536	Hagstrom, *et al*.[20]
Exon 14	c.1381C>G; p.(Leu461Val)/ c.1381C>G; p.(Leu461Val)	juvenile RP	1/86	1/86	den Hollander, *et al*.[24]
Exon 14	**c.1445G>A; p.(Arg482Gln)/ c.1445G>A; p.(Arg482Gln)**	**early onset arRP**	**1/2**	**6/33**	**This study**
Exon 14	c.1466A>G; p.(Lys489Arg)/ c.1466A>G; p.(Lys489Arg)	early onset arRP, arRP	4/5, 1/171	19/23, 1/171	Iqbal, *et al*.,[15] Gu, *et al*.[19]***
Intron 14	c.1495+1G>A; p.(?)/ c.1495+1G>A; p.(?)	arRP	2/2	33/33	Banerjee, *et al*.[21]
Intron 14	c.1495+2dup; p.(?)/c.1495+2dup; p.(?)	early onset arRP	1/1	3/3	Abbasi, *et al*.[26]
Intron 14	c.1496-6C>A; p.(?)/c.1496-6C>A; p.(?)	arRP	1/171	1/171	Gu, *et al*.[19]
Exons 14, 15	c.1444C>T; p.(Arp482Trp)/ c.1511_1521del; p.(Leu504Profs*141)	early onset arRP	1/1	5/5	den Hollander, *et al*.[50]
Exon 15	c.1582_1587dup; p.(Phe528_Ala529dup)/ c.1582_1587dup; p.(Phe528_Ala529dup)	LCA	1/1	7/7	Mataftsi, *et al*.[25]****

**Variant labelled as c.937delC. ***Same change at protein level but labelled as c.1502G>A at the cDNA level. ****Change labelled as c.1593_1594TTCGCC (FA531-532dup). The variants identified in this study are marked in bold. Patients with visual loss, nystagmus and night blindness after the age of six months but not later than six years are diagnosed as juvenile RP.[24]

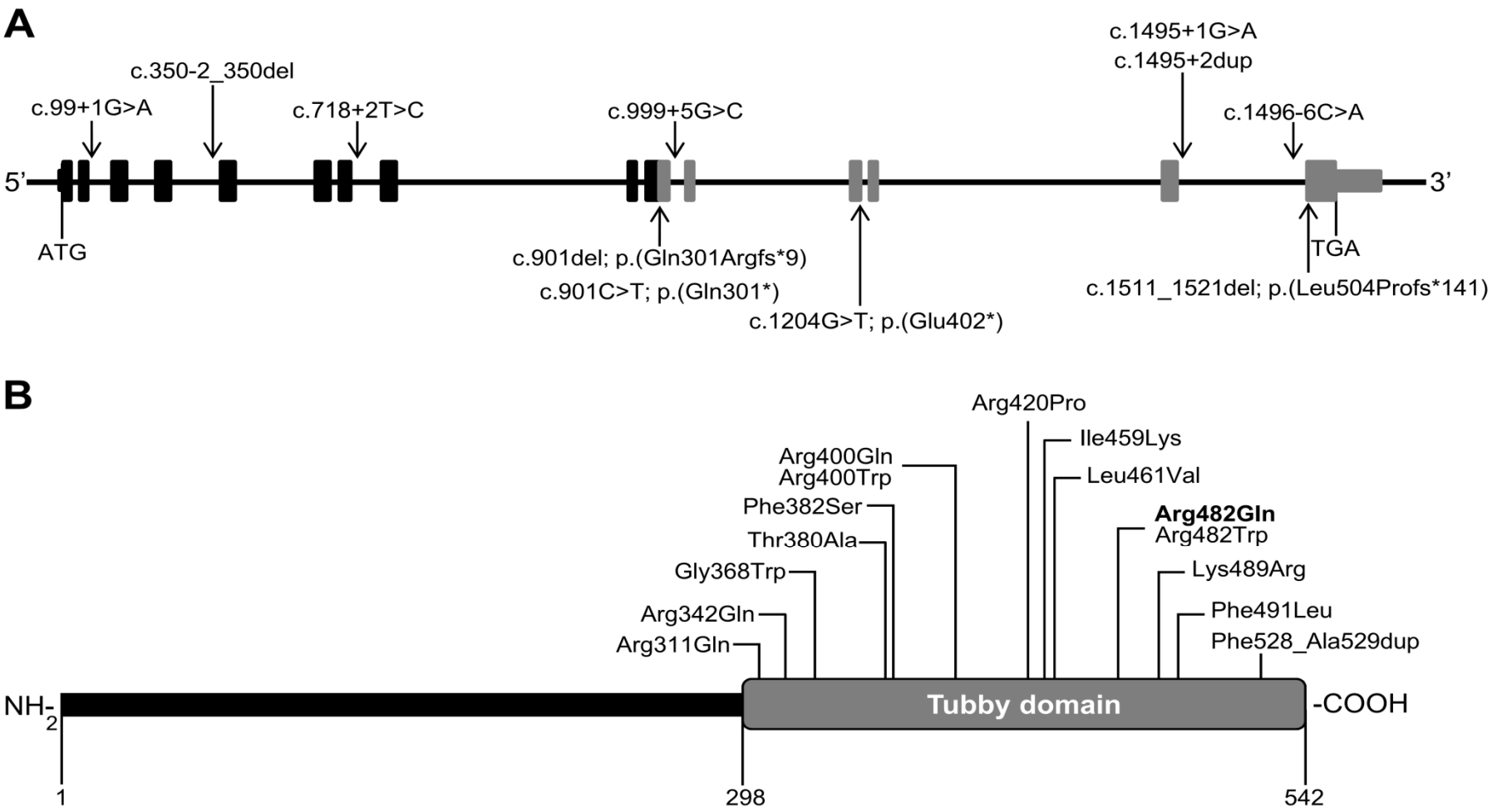

Figure 2.6 *TULP1* gene, protein structure, and pathologic variants identified in patients with LCA and arRP. A: Gene organization and distribution of splice site variants (above the gene) and nonsense and frame-shift variants (below the gene). **B**: Protein domain structure of tubby-like protein 1 (TULP1) showing missense changes and a two-amino acid duplication in the tubby, C-terminal domain. The novel missense variant is indicated in bold.

Acknowledgments

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Chapter 3

Novel mutations in *RDH5* cause fundus albipunctatus in two consanguineous Pakistani families

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ABSTRACT

Purpose: To identify the underlying genetic causes of fundus albipunctatus (FA), a rare form of congenital stationary night blindness that is characterized by the presence of white dots in the midperiphery of the retina and delayed dark adaptation, in Pakistan.

Methods: Two families with FA were identified by fundus examination, and genome-wide single nucleotide polymorphism genotyping was performed for two individuals from family A and six individuals from family B. Genotyping data were subsequently used to identify the identical homozygous regions present in the affected individuals of both families using the online homozygosity mapping tool Homozygosity Mapper. Candidate genes selected from the homozygous regions were sequenced.

Results: Three identical homozygous regions were identified in affected persons of family A (on chromosomes 8, 10, and 12), whereas a single shared homozygous region on chromosome 12 was found in family B. In both families, the homozygous region on chromosome 12 harbored the retinol dehydrogenase 5 (*RDH5*) gene, in which mutations are known to be causative of FA. *RDH5* sequence analysis revealed a novel five base pair deletion, c.913_917delGTGCT; p.(Val305Hisfs*29), in family A, and a novel missense mutation, c.758T>G; p.(Met253Arg), in family B.

Conclusions: We identified two novel disease-causing *RDH5* mutations in Pakistani families with FA, which will improve diagnosis and genetic counseling, and may even lead to treatment of this disease in these families.

3.1. Introduction

Fundus albipunctatus (FA; OMIM:136880), or flecked retina disease, was described for the first time by Lauber.[1] FA is a rare form of congenital stationary night blindness and is characterized by the presence of typical white dots on the whole fundus or concentrated in the midperipheral region of the retina, with or without macular involvement, and a delay in dark adaptation. The inheritance pattern of FA is autosomal recessive.[2-5] In one family, a male and his two daughters showed FA, which could be due to autosomal dominant or pseudo dominant (i.e., autosomal recessive) inheritance.[6] Mutations in three genes–retinol dehydrogenase 5 (*RDH5*), retinaldehyde-binding protein 1 (*RLBP1*), and retinal pigment epithelium–specific protein (*RPE65*) - are known to be associated with FA.[7-10] Retinitis punctata albescens has similar phenotypic characteristics but is progressive in nature and is mostly caused by mutations in *RLBP1*.[8]

FA-causing mutations were first identified in *RDH5*, which is expressed predominantly in the retinal pigment epithelium (RPE).[7] *RDH5* encodes an enzyme that is part of the visual cycle, which involves a series of specialized enzymes and retinoid binding proteins that are essential for the regeneration of the 11-*cis* retinal chromophore.[11-14] RDH5 consists of 318 amino acids and is highly conserved among different species.[15] Within the RPE cells, RDH5 resides in the smooth endoplasmic reticulum[16] where it is principally involved in chromophore regeneration by catalyzing the final step in the biosynthesis of 11-*cis* retinal.[7,17-20]

The current study explores the molecular mechanisms behind FA in Pakistani families, using high-density single nucleotide polymorphism (SNP) microarrays and sequence analysis of known FA genes located in the identified homozygous regions. Using this approach, we identified two novel mutations in *RDH5* in two families with FA.

3.2. Methods

3.2.1. Approval of the study

Approval for this study was granted by the Ethics Committee/Institutional Review Board of Shifa College of Medicine/Shifa International Hospital, Islamabad. Signed informed consent was obtained from members of both families participating in the current study.

3.2.2. Family collection and clinical evaluation

Families A and B (Figure 3.1) reside in remote areas of Pakistan and were part of a cohort of 83 families with retinitis pigmentosa and associated retinal diseases. Blood samples were collected from affected and normal individuals of both families and DNA was extracted by a standard protocol.[21] Pedigrees were drawn using HaploPainter.[22] Both families were clinically evaluated by fundus examination; in addition, electroretinography (ERG) measurements were recorded for family A.

3.2.3. Homozygosity mapping analysis

All affected individuals from both families and one healthy person from family B were subjected to high-density HumanOmniExpress (>700 K; Illumina Inc., San Diego, CA) SNP microarray analysis. Genotyping data were analyzed with the online tool Homozygosity Mapper.[23] Haplotypes of affected and normal individuals were compared in each family to identify the identical homozygous regions shared by all affected individuals.

3.2.4. Primer design and *RDH5* sequence analysis

The online tool Primer3[24] was used to design PCR primers (Table 3.1). The five exons of RDH5, including their flanking exon-intron boundaries, were amplified by PCR using standard conditions and reagents. PCR-amplified exonic fragments were electrophoretically separated on 2% agarose gels containing ethidium bromide and DNA bands were visualized under ultraviolet transillumination. PCR clean-up purification plates (NucleoFast® 96 PCR; Cat. No. 743100.10, Macherey-Nagel, Düren, Germany) were used to purify the amplified fragments according to the manufacturer's protocol. Briefly, 20 µl of each amplified PCR product was transferred to Nucleofast 96 PCR plate. Wells were filled up to 100 µl volume with RNase-free water to ensure the uniform loading. Contaminants were removed by ultrafilteration with the help of a vacuum apparatus for 10 min. Thirty µl of RNase-free water was poured in each well and DNA was recovered by thorough mixing with a multi-channel pipette. Sanger sequencing was then performed with Big Dye Terminator version 3 and analyzed on a 3730 DNA analyzer (Applied Biosystems, Inc., Foster City, CA).

Vector NTI Advance (TM) 2011 software from Invitrogen Corporation (Carlsbad, CA) was used to analyze the sequencing results of *RDH5* (NM_001199771.1) exons.

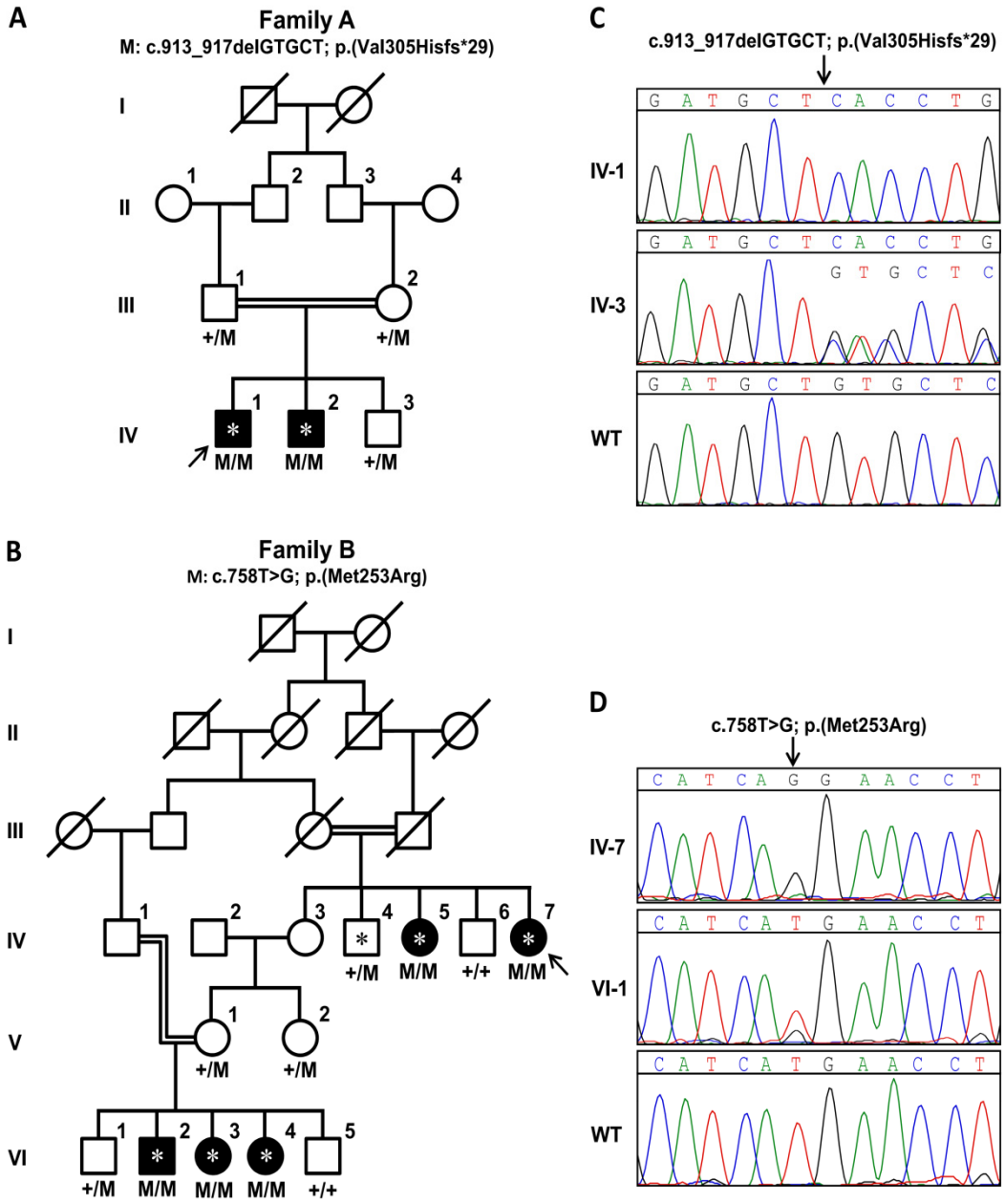

Figure 3.1 Pedigrees and sequencing results. A: Segregation of the mutation in family A. **B:** Segregation of the mutation in family B. **C** and **D:** Sequence electropherograms of affected individuals carrying homozygous variants (upper panels) and unaffected heterozygous carriers (middle panels) of families A (C) and B (D), along with the results of a control individual (wild-type [wt], lower panels). Arrows point to the probands; individuals tested with single nucleotide polymorphism (SNP) microarrays are indicated with asterisks.

3.2.5. In silico analysis

Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html), Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/), and Mutation Taster[25] were used to assess the possible pathological nature of the missense variant

identified in this study. Project HOPE[26] (http://www.cmbi.ru.nl/hope/home) was used to analyze and predict the structural variations in mutant RDH5.

Table 3.1 Primer sequences of *RDH5* exons.

Exon	Forward primer (5'→3')	Reverse primer (5'→3')	length (bp)
1	CTAGGCAAATCTGGCCTCTG	GGTCCACCTCAGAGTTGTGG	396
2	GGAAAGGGCTTGAGGGC	GACTGTGGGGATCAGGACAC	450
3	CTCCCAGGAAGAAGAGGGAG	CACCTCTGCTGGCCCAC	399
4	ATGTCCCTCAAAGTCCCCTC	AGGCTTATGCAGGACTGGC	301
5	GGCCCCAGAAGACAGTACC	CGTGCAGCTGTAGATGTGAG	589

3.2.6. Amino acid conservation

RDH5 protein sequences from different species including human (*Homo sapiens*, ENSP00000257895), macaque (*Macaca mulatta*, ENSMMUP00000017380), mouse (*Mus musculus*, ENSMUSP00000026406), dog (*Canis familiaris*, ENSCAFP00000000084), cow (*Bas taurus*, ENSBTAP00000056512), cat (*Felis catus*, ENSFCAP00000012945), tetraodon (*Tetraodon nigroviridis*, ENSTNIP00000022889), and round worm (*Caenorhabditis elegans*, F35B12.2) were aligned using Vector NTI Advance™ 2011 to check the evolutionary conservation of the substituted amino acid in RDH5.

3.3. Results

3.3.1. Clinical studies

Initial symptoms of visual complaints in patients from both families were observed from early childhood. Fundus examination of affected individuals revealed the presence of white dots typical of FA in the midperiphery of the retina (Figure 3.2; Table 3.2). ERG responses of cone and rod photoreceptors were diminished in affected individual IV-1 of family A (Table 3.3). This individual had daytime vision problems, which confirms that cone photoreceptors were also affected. Macular degeneration was also observed in individual IV-1 of family A and individual IV-7 of family B. ERG results were not available for family B. The visual acuity (VA) of affected individual IV-7 of family B was different from the VAs of other individuals (VI-2, VI-3) of this family, and the density of white dots was also variable, which indicates intrafamilial phenotypic variability. Affected individuals of family B had normal daytime vision.

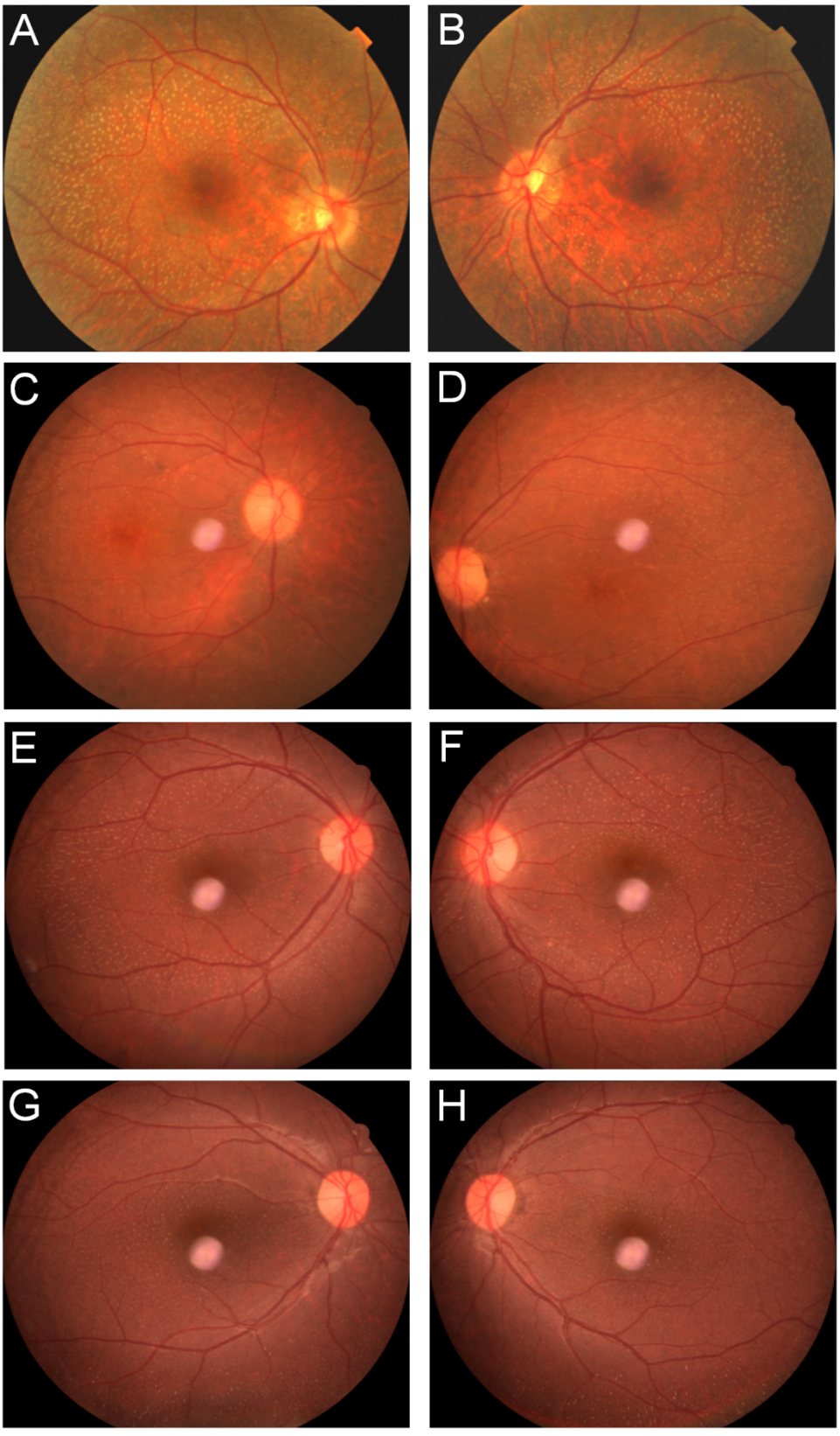

Figure 3.2 Fundus photographs of affected individuals from both families. A, B: Right and left eye, respectively, of affected individual IV-1 of family A (see arrow, Figure 3.1A). **C, D:** Right and left eye, respectively, of affected individual IV-7 of family B (see arrow, Figure 3.1B). **E, F:** Right and left eye, respectively, of affected individual VI-2 of family B. **G, H:** Right and left eye, respectively, of affected individual VI-3 of family B.

Table 3.2 Clinical features of affected individuals in both families.

Individual	Age (years)	VA (RE, LE)	Fundus Phenotype	RPE Degeneration	Retinoscopy
Family A (IV-1)	35	6/12, 6/12	White dots, macular degenerative changes	Yes	ND
Family B (IV-7)	45	6/18, 6/12	White dots, macular degenerative changes	Yes	HM
Family B (VI-2)	17	6/6, 6/6	White dots, macula healthy	No	LHM
Family B (VI-3)	10	6/6, 6/6	White dots, macula healthy	No	LHM

HM, hypermetropia; LE, left eye; LHM, low hypermetropia; RE, right eye; ND, not determined; RPE, retinal pigment epithelium; VA, visual acuity.

Table 3.3 ERG responses of affected individual IV-1 of Family A in comparison with ERG responses of a control individual.

Measured parameters using monopolar electrodes	Adaptation	Flash strength (cd.s/m^2)	Proband Family A	Control	Normal values (Age=40 years)
Scotopic 25 dB b-wave amplitude (μV)	Dark	0.01	45.1	173.20	>141
Scotopic 0 dB b-wave amplitude (μV)	Dark	3.0	149.1	496.80	>387
Oscillatory potential amplitude (μV)	Dark	3.0	80.3	123.90	>75
Photopic 0 dB b-wave amplitude (μV)	Light	3.0	70.7	80.80	>82
Photopic 30 Hz flicker amplitude (μV)	Light	3.0	49.5	55.90	>56

Age of affected individual at the time of investigation was 35 years

3.3.2. Genetic studies

In family A, three homozygous regions were identified that were shared by the affected persons (Figure 3.3A). The largest homozygous region spanned 24.5 Mb (hg19: 3.3–27.8 Mb; flanked by SNPs rs4881131 and rs10764698) on chromosome 10. The second and third homozygous regions were 10.5 Mb (hg19: 46.4–56.9 Mb; flanked by rs11183300 and rs7314300) and 8.1 Mb (hg19: 25.9–34.0 Mb; flanked by rs9521585 and rs9555687) in length, and were located on chromosomes 12 and 8, respectively. The second largest region (10.5 Mb) on chromosome 12 harbored the FA-associated gene *RDH5*. *RDH5* sequence analysis identified a novel homozygous 5 bp deletion (c.913_917delGTGCT; p.(Val305Hisfs*29)) in family A (Figure 3.1C).

The mutation c.913_917delGTGCT; p.(Val305Hisfs*29) segregated in family A (Figure 3.1A) was consistent with an autosomal recessive inheritance pattern. Both affected individuals carried this mutation in a homozygous state, while both parents and an unaffected brother carried this variant in a heterozygous state. The mutation causes a frameshift in the open reading frame and results in the replacement of the last 14 amino acids of the RDH5 protein by 28 aberrant amino acids. This mutation is predicted to affect part of the transmembrane domain and elongate the cytosolic C-terminal tail. As this deletion is located in the last exon of *RDH5*, nonsense-mediated decay of the mutant mRNA is not predicted.

In family B homozygosity mapping revealed an 8.9 Mb (hg19: 52.6–61.5 Mb) homozygous segment (Figure 3.3B) flanked by SNPs rs1894035 and rs1395538, encompassing the *RDH5* gene. *RDH5* sequence analysis revealed a novel homozygous missense mutation (c.758T>G; p.(Met253Arg)) in this family. Segregation analysis confirmed that all affected individuals were homozygous for the mutation c.758T>G; p.(Met253Arg) (Figure 3.1B), suggesting that this variant may be disease causing. The methionine at position 253 is a highly conserved amino acid residue among different species (Figure 3.4), and c.758T is an evolutionarily highly conserved nucleotide with a PhyloP score of 4.40. SIFT predicted p.(Met253Arg) to be a deleterious (score: 0.05) mutation, PolyPhen-2 classified this mutation as probably damaging (score: 0.992), and Mutation Taster predicted this mutation to be disease causing. Structural analysis showed that there was a difference in charge and size of the wild-type Met253 and the mutant Arg253. The wild-type residue is uncharged, whereas the mutant residue is positively charged. The wild-type residue is buried in the alpha helix and the mutant residue introduces a charge in this buried residue in the core of the protein or protein complex,

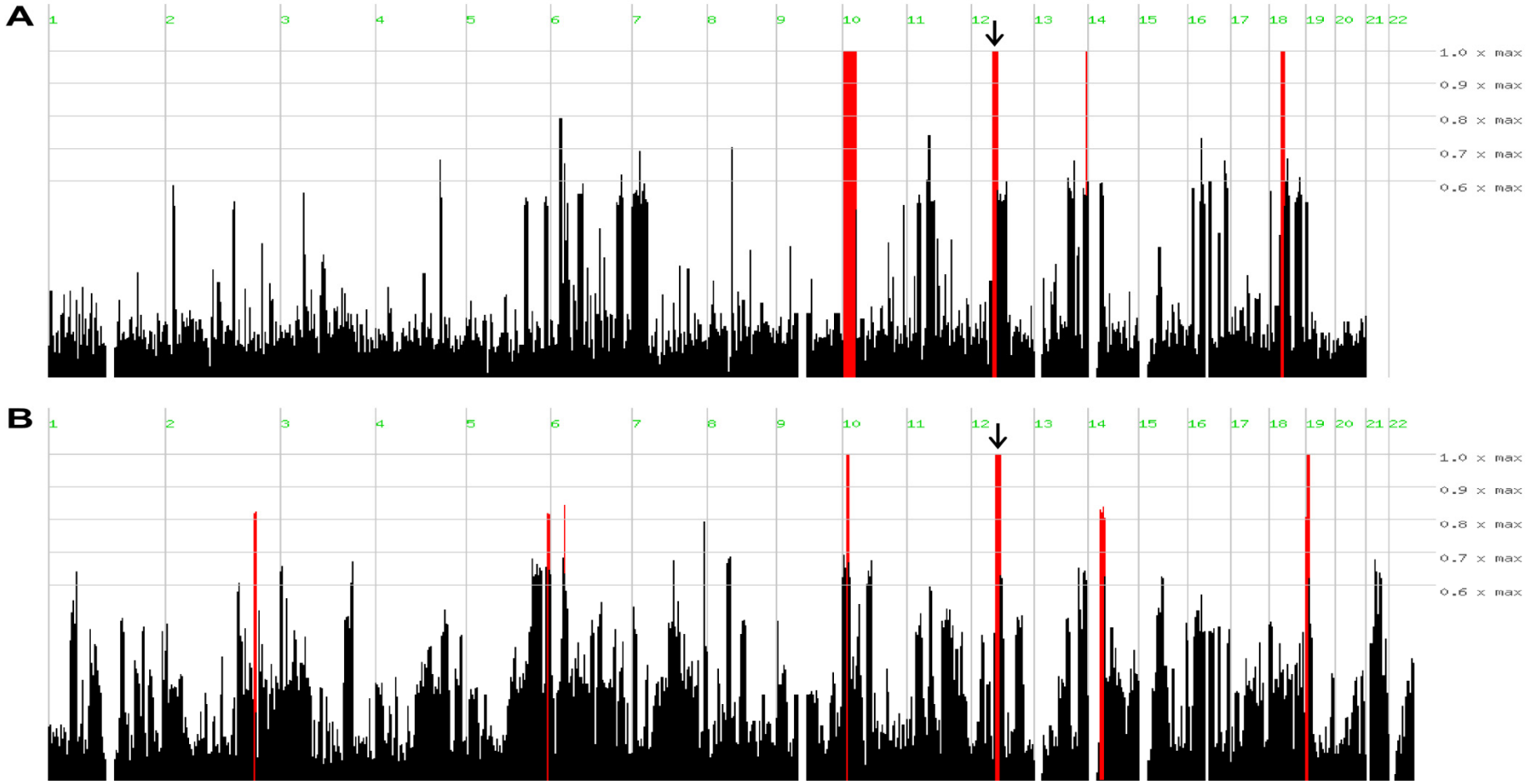

Figure 3.3 Homozygosity mapping results. A: Plot of homozygous regions identified in affected individuals in family A using Homozygosity Mapper analysis. **B:** Plot of homozygous regions identified in affected individuals in family B using Homozygosity Mapper analysis. The red lines indicate homozygous regions shared by affected individuals in each family. The arrows indicate the homozygous regions that harbor *RDH5*.

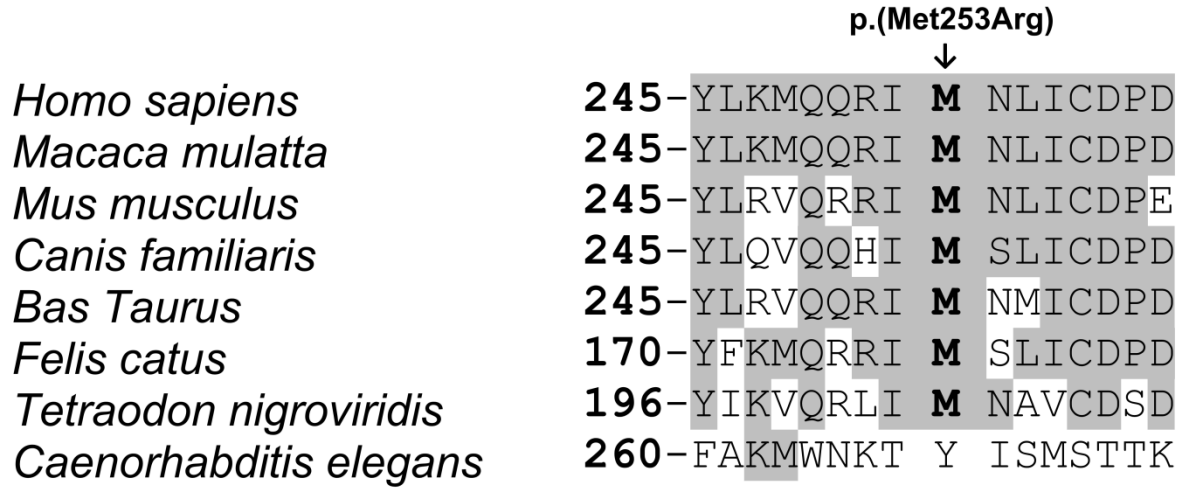

Figure 3.4 Conservation of amino acids 245–260 of RDH5 in different species. Gray shading indicates amino acids that are identical to human RDH5 amino acids.

which can lead to misfolding of the protein. The mutant residue is bigger and probably will not fit in the core of the protein. The hydrophobicities of the wild-type and mutant residue also differ, and therefore, this mutation is likely to cause the loss of hydrophobic interactions in the core of the protein.

Ethnically matched control samples were not tested for these mutations; however, neither variant was found in dbSNP or in 1000 Genomes.

3.4. Discussion

In this study, we have identified two novel disease-causing mutations in *RDH5* in two unrelated consanguineous families with FA. Both families exhibited typical FA, as was evident from the presence of typical white dots in the midperipheral regions of the retina. In both families, the older patients–IV-1 in family A and IV-7 in family B–had macular degeneration, which might suggest a progressive disease course in these families.

Including our findings, 36 different mutations in *RDH5* associated with FA have been identified to date.[7,27-48] FA patients carrying *RDH5* mutations exhibit high phenotypic variability, ranging from non-progressive to progressive disease, a variable VA, variation in the density of white dots, and occasionally macular involvement. FA with or without cone dystrophy has also been reported with varying degrees of severity.[30,37,48] A total of 85 FA patients from 68 different families carrying *RDH5* mutations have been identified globally (Table 3.4). These persons were found to exhibit a high variability in phenotype, but the presence of white dots was a common feature. In comparing the different phenotypes and

genotypes associated with *RDH5*, it is difficult to establish a valid and clear-cut genotype-phenotype correlation.

RDH5 is a transmembrane enzyme with a membrane-embedded N-terminal domain, a catalytic ectodomain, a C-terminal transmembrane domain, and a cytosolic tail.[16] The topology of retinol dehydrogenases has been controversial as human retinal reductase-1[49] and mouse retinol dehydrogenase-1[50] have been reported to have a membrane-embedded N-terminal domain but no C-terminal transmembrane segment, which supports the presence of a cytosolic ectodomain. RDH5 was suggested to have a cytosolic ectodomain without any C-terminal transmembrane domain.[50] However, another retinol dehydrogenase, *cis*-retinol/androgen dehydrogenase 1 (CRAD1), has been described in detail to have a RDH5-like structure with both a luminal ectodomain and cytosolic C-terminal domain, and a similar topology has been suggested for most of the retinol dehydrogenases.[51] The frameshift mutation p.(Val305Hisfs*29) identified in family A is located in the C-terminal transmembrane domain, while the missense mutation p.(Met253Arg) is located in the catalytic ectodomain of RDH5 (Figure 3.5). As the C-terminal transmembrane region is necessary to retain CRAD1 in the endoplasmic reticulum,[51] the RDH5 mutation p.(Val305Hisfs*29) might affect the endoplasmic reticulum localization of RDH5. Moreover, an elongated C-terminal cytosolic tail might also create problems in the proper functioning of RDH5, as the C-terminus is thought to play a role in enzymatic activity and localization of CRAD1 and RDH5.[51]

Structural analysis of RDH5 performed with Project HOPE suggests that the missense mutation p.(Met253Arg) may cause misfolding of the RDH5 protein because of the loss of hydrophobic interactions in the core of the mutant protein. Misfolding of the mutant protein may cause it to degrade.[52-54] Absence of RDH5 leads to the accumulation of 11-*cis* retinol[20] in the RPE, and a reduction of 11-*cis* retinal in the photoreceptors, which in turn might result in the malfunctioning of rod and cone photoreceptor cells.

RDH5-associated disease can be prevented with proper genetic counseling of carriers of *RDH5* mutations, and persons with this disease can be treated with 9-*cis*-β-carotene supplementation. $Rdh^{-/-}$ mice were successfully treated with 9-*cis* retinal,[55] and 9-*cis*-β-carotene was given to FA patients leading to major visual improvements;[56] 9-*cis*-β-carotene is converted to 9-*cis* retinal,[57,58] which is more stable than 11-*cis* retinal.[59] The higher stability of opsin bound to 9-*cis* retinal slows down the visual cascade and thus minimizes the toxicity of

Table 3.4 *RDH5* mutations causing fundus albipunctatus.

Exon/intron	Allele 1	Allele 2	Phenotype	Families	Cases	Reference
Exon 2	Wt	c.55A>G; p.(Arg19Gly)	DWD	1	1	Sergouniotis, *et al*.[48]
Exon 2, 4	c.95del; p.(Phe32Serfs*29)	c.712G>T; p.(Gly238Trp)	WD, MA	1	1	Schatz, *et al*.[47]
Exon 2, 3	c.98T>A; p.(Ile33Asn)	c.469C>T; p.(Arg157Trp)	WD	1	1	Ruther, *et al*.[39]
Exon 2, 4	c.98T>C; p.(Ile33Thr)	c.712G>T; p.(Gly238Trp)	DWD, RPED	1	1	Sergouniotis, *et al*.[48]
Exon 2	c.103G>A; p.(Gly35Ser)	c.103G>A; p.(Gly35Ser)	WD, CD, BE	1,1,1	2,1,1	Nakamura, *et al*.,[30] Wada, *et al*.,[32] Nakamura, *et al*.,[37] Querques, *et al*.[46]
Exon 2, 5	c.103G>A; p.(Gly35Ser)	c.928delinsGAAG; p.(Leu310delinsGluVal)	WD	1, 2	1, 2	Nakamura, *et al*.,[30] Nakamura, *et al*.,[37] Niwa, *et al*.[41]
Exon 2, 5	c.124C>T; p.(Arg42Cys)	c.928delinsGAAG; p.(Leu310delinsGluVal)	WD	1	1	Niwa, *et al*.[41]
Exon 2	c.129del; p.(Leu44Trpfs*17)	c.129del; p.(Leu44Trpfs*17)	WD	1	1	Driessen, *et al*.[33]
Exon 2, 5	c.211_214dup; p.(Ala72Glyfs*15)	c.801C>G; p.(Cys267Trp)	WD	1	1	Driessen, *et al*.[33]
Exon 2, 4	c.218C>T; p.(Ser73Phe)	c.712G>T; p.(Gly238Trp)	WD	1	1	Yamamoto, *et al*.[7]

Intron 2, 4	c.310+1G>A; p.(?)	c.712G>T; p.(Gly238Trp)	DWD	1	1	Sergouniotis, *et al*.[48]
Exon 3	c.319G>C; p.(Gly107Arg)	c.319G>C; p.(Gly107Arg)	WD, MD, SRP	1, 1	2, 1	Hotta, *et al*.,[35] Sato, *et al*.[40]
Exon 3, 5	c.319G>C; p.(Gly107Arg)	c.928delinsGAAG; p.(Leu310delinsGluVal)	WD, BE	1	1	Nakamura, *et al*.[30]
Exon 3	c.346_347insGCA; p.(Gly116_Ile117insSer)	c.346_347insGCA; p.(Gly116_Ile117insSer)	DWD, RPED	1	1	Sergouniotis, *et al*.[48]
Exon 3, 4	c.346G>C; p.(Gly116Arg)	c.710A>C; p.(Tyr237Ser)	NWD	1	1	Sergouniotis, *et al*.[48]
Exon 3	c.382G>A; p.(Asp128Asn)	c.382G>A; p.(Asp128Asn)	WD	1	1	Schatz, *et al*.[47]
Exon 3, 4	c.382G>A; p.(Asp128Asn)	c.712G>T; p.(Gly238Trp)	WD	1	1	Iannaccone, *et al*.[43]
Exon 3, 5	c.394G>A; p.(Val132Met)	c.839G>A; p.(Arg280His)	WD, CD, MD	1, 1, 3	1, 2, 3	Nakamura, *et al*.,[30] Nakamura, *et al*.,[37] Nakamura, *et al*.,[38] Niwa, *et al*.[41]
Exon 3, 5	c.416G>T; p.(Gly139Val)	c.955T>C; p.(*319Argext*33)	DWD, RPED	1	1	Sergouniotis, *et al*.[48]
Exon 3	c.470G>A; p.(Arg157Gln)	c.470G>A; p.(Arg157Gln)	DWD, RPED	1	1	Sergouniotis, *et al*.[48]
Exon 3, 4	c.470G>A; p.(Arg157Gln)	c.712G>T; p.(Gly238Trp)	WD	1	1	Hajali, *et al*.[45]

Exon 3	c.490G>T; p.(Val164Phe)	c.490G>T; p.(Val164Phe)	WD, MA	1	1	Yamamoto, *et al*.[36]
Exon 3, 5	c.530T>G; p.(Val177Gly)	c.839G>A; p.(Arg280His)	WD	1	1	Kuroiwa, *et al*.[29]
Exon 3, 5	c.530T>G; p.(Val177Gly)	c.928_930delinsGAAGTT; p.(Leu310delinsGluVal)	WF	1	1	Hayashi, *et al*.[42]
Exon 4	c.625C>T; p.(Arg209*)	c.625C>T; (p.Arg209*)	WD	1	1	Schatz, *et al*.[47]
Exon 4, 5	c.689_690delinsGG; p.(Pro230Arg)	c.928delinsGAAG; p.(Leu310delinsGluVal)	WD	1	1	Wang, *et al*.[44]
Exon 4	c.712G>T; p.(Gly238Trp)	c.712G>T; p.(Gly238Trp)	WD, DWD, DWF	1, 1	2, 2, 1	Yamamoto, *et al*.,[7] Gonzalez-Fernandez, *et al*.,[27] Sergouniotis, *et al*.[48]
Exon 4, 5	c.718dup; p.(Ala240Glyfs*19)	c.841T>C; p.(Tyr281His)	WD, BE, MD	1, 1	1, 1	Nakamura, *et al*.,[30] Niwa, *et al*.[41]
Exon 5	**c.758T>G; p.(Met253Arg)**	**c.758T>G; p.(Met253Arg)**	**WD, MD**	**1**	**5**	**This study**
Exon 5	c.791T>G; p.(Val264Gly)	c.791T>G; p.(Val264Gly)	WD	1	3	Hirose, *et al*.[28]
Exon 5	c.824_825del; p.(Arg275Profs*60)	c.824_825del; p.(Arg275Profs*60)	DWD, DWF, RPED	1	1	Sergouniotis, *et al*.[48]
Exon 5	c.839G>A; p.(Arg280His)	c.880G>C; p.(Ala294Pro)	WD, MD	1	2	Gonzalez-Fernandez, *et al*.,[27]

Exon 5	c.839G>A; p.(Arg280His)	c.928delinsGAAG; p.(Leu310delinsGluVal)	WD	1, 1, 2	1, 1, 2	Nakamura, *et al*.,[30] Nakamura, *et al*.,[37] Sato, *et al*.,[40] Niwa, *et al*.[41]
Exon 5	c.841T>C; p.(Tyr281His)	c.928delinsGAAG; p.(Leu310delinsGluVal)	WD, MD	1, 1	1, 1	Nakamura, *et al*.,[34] Niwa, *et al*.[41]
Exon 5	c.880G>C; p.(Ala294Pro)	c.880G>C; p.(Ala294Pro)	WD	1	1	Schatz, *et al*.[47]
Exon 5	**c.913_917del; p.(Val305Hisfs*29)**	**c.913_917del; p.(Val305Hisfs*29)**	WD, MD	**1**	**2**	**This study**
Exon 5	c.928delinsGAAG; p.(Leu310delinsGluVal)	c.928delinsGAAG; p.(Leu310delinsGluVal)	WD, BE, PP	1, 4, 1, 1, 4, 6	1, 4, 2, 1, 6, 6	Hirose, *et al*.,[28] Nakamura, *et al*.,[30] Wada, *et al*.,[31] Nakamura, *et al*.,[37] Niwa, *et al*.[41]

BE, bull's eye; CD, cone dystrophy; DWD, deep whitish dots; DWF, deep whitish flecks; MA, macular atrophy; MD, macular dystrophy; NWD, no white dots; PP, photophobia; RPED, retinal pigment epithelium degeneration; SRP, sectorial retinitis pigmentosa; WD, white dots; WF, white flecks. Mutations identified in this study are in bold

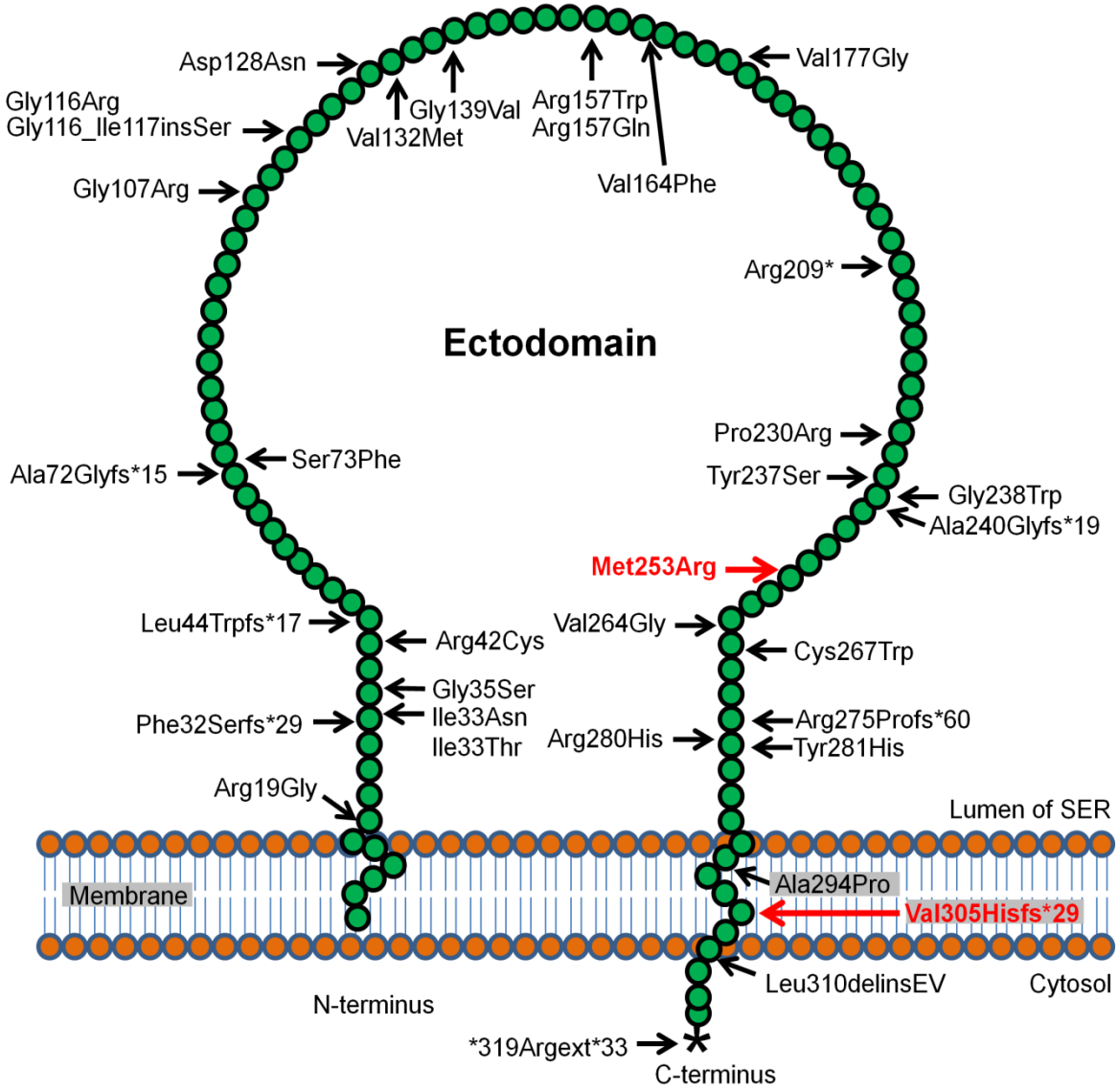

Figure 3.5 Schematic representation of RDH5 and all mutations thus far published. The membrane-embedded N-terminus consists of 18 amino acids and the ectodomain, present in the lumen of the smooth endoplasmic reticulum (SER), spans amino acids 19 – 288. A C-terminal membrane-spanning domain encompasses amino acids 289 – 310, and a small cytosolic tail of eight amino acids resides in the cytosol of retinal pigment epithelium (RPE) cells. Both missense and protein-truncating mutations are distributed across the entire protein. Mutations identified in this study are indicated in red.

accumulating by-products in the visual cycle.[55,60,61] In the rod-photoreceptor outer segments 9-*cis* retinol will be converted to all-trans retinal during bleaching. This is subsequently reduced to all-trans retinol and, in the RPE, all-trans retinol is isomerically converted to 9-*cis*, 11-*cis*, and 13-*cis* retinol. A stereospecific enzyme, 9-*cis* retinol dehydrogenase, is reported to be involved in the synthesis of 9-*cis* retinoic acid by oxidizing 9-*cis* retinol,[62] and 9-*cis* retinal treatment is suggested to induce the endogenous synthesis of 11-*cis* retinal by its interaction with the retinoid X nuclear receptor.[56,59,63]

Based on our and other studies, we estimate that FA contributes to approximately 2% (4/208) of families with retinal dystrophy in Pakistan and a total of 17 patients have been identified with FA.[9] Two FA families have been reported to carry *RLBP1* mutations,[9] while two other families with FA have *RDH5* mutations (this study). In the current study, we have identified seven additional FA patients who are candidates for 9-*cis*-β-carotene therapy.

In conclusion, we have identified two novel disease-causing mutations, c.913_917delGTGCT; p.(Val305Hisfs*29) and c.758T>G; p.(Met253Arg), in two Pakistani families with FA. Our study expands the current mutation spectrum of *RDH5* and contributes to the existing body of knowledge. In addition, this study will help clinicians to improve the diagnosis of FA by differentiating FA from retinitis punctata albescens, providing genetic counseling and prescribing the correct treatment to patients.

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Chapter 4

Exome sequencing identifies a novel and a known *BBS1* mutation in Pakistani families with Bardet-Biedl syndrome

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ABSTRACT

Purpose: To determine the genetic cause of Bardet-Biedl syndrome (BBS) in two consanguineous Pakistani families.

Methods: Clinical characterization of the affected individuals of both families was done by ophthalmic examination, electroretinography, electrocardiography, liver and renal profile. A total of 17 genes are known to be associated with BBS, so exome sequencing was preferred over candidate gene sequencing. One affected individual from both families was selected for exome sequencing. Identified variants were confirmed by Sanger sequencing.

Results: Retinitis pigmentosa, obesity and learning difficulties were present in affected individuals of both families. In family A, a sixth finger (polydactyly) of the proband's sister was removed by a surgical operation leaving a scar on the little finger. Polydactyly was also present in both affected individuals from family B. All diagnostic symptoms were characteristic of BBS in both families. In both affected individuals from family A, exome sequencing identified a novel homozygous mutation (c.47+1G>T) in *BBS1* that inactivates the splice donor site at the end of exon 1. In family B, a previously reported mutation, c.442G>A; p.(Asp148Asn), was detected.

Conclusions: Exome sequencing is an efficient technique for the identification of mutations in genetically heterogeneous diseases. In addition, intra-familial phenotypic variability in family A argues for the modifying effect of some other still unknown modifier alleles.

Key words: *BBS1*; phenotypic variability; polydactyly; splice-site mutation; exome sequencing.

4.1. Introduction

Bardet-Biedl syndrome (BBS) (MIM: 209900) is invariantly characterized by rod-cone dystrophy, and at least three additional non-ocular features such as intellectual disability, obesity, polydactyly, hypogonadism or renal anomalies as primary manifestations. In the absence of one of these four primary clinical feature, the diagnosis of BBS is made when at least two secondary features are observed, including hepatic fibrosis, diabetes mellitus, reproductive and developmental abnormalities, growth retardation, speech delays, or cardiovascular problems.[1]

BBS has been classified as a ciliopathy[2] and is inherited mostly in an autosomal recessive pattern although digenic (tri-allelic) inheritance has also been reported.[3-6] Some studies have also demonstrated the epistatic effects of a third protective allele,[7,8] while variants in *RPGRIP1L* have been reported as a modifier of the BBS phenotype.[9] In only two studies, a recessive mode of inheritance for BBS has been argued against.[5,10] Very recently, a spectrum of phenotypes ranging from full-blown BBS to non-syndromic RP was found to be associated with the hypomorphic *BBS1* missense mutation p.(Met390Arg).[11]

BBS is a severe disorder with the highest prevalence of 1/3,700 in the Faroe Islands.[12] While in the rest of the world its prevalence varies from 1/13,000 in Newfoundland,[13] 1/17,000 in the Kuwaiti population[14] and 1/65,000 in other Arab countries,[15] it is rare in the European population with a prevalence range of 1/125,000 in the United Kingdom[16] to 1/160,000 in Switzerland.[17]

BBS is genetically heterogeneous, as mutations in 17 different genes have been identified so far.[18] The *BBS1* gene (MIM: 209901) is located on the long arm of chromosome 11 and consists of 17 coding exons. The gene is expressed in many tissues including fetal, testicular, retinal, adipose, cardiac, skeletal and pancreatic cells, with the highest expression in kidney.[19] BBS1 is part of the BBSome complex that includes BBS1, BBS2, BBS4, BBS5, BBS7, BBS8 and BBS9. Proteins in this complex are thought to be involved in ciliogenesis, because of their function in membrane trafficking in the primary cilium.[20]

The current study was designed to find the molecular basis of BBS in two Pakistani families (family A and B) using exome sequencing. A novel splice donor site mutation c.47+1G>T in *BBS1* was identified in family A, and a previously reported *BBS1* mutation, c.442G>A; p.(Asp148Asn),[21] was identified in family B.

4.2. Materials and Methods

4.2.1. Ethics committee/Institutional review board approval

Approval for this study was granted by the ethics committee/institutional review board of Shifa College of Medicine/Shifa International Hospital, Islamabad. Written informed consent was obtained from both families before starting the study. In addition, the study conformed to the tenants of the declaration of Helsinki.

4.2.2. Clinical examination

Clinical examination included ophthalmic examination, electroretinography, echocardiography, liver function test and renal profile. Blood sugar was also measured to determine the presence or absence of diabetes mellitus.

4.2.3. Blood sampling and DNA preparation

Both families (Figure 4.1A, B) were recruited from the central part of Punjab. Venous blood of affected and normal individuals of both families was drawn by venipuncture and collected in acid citrate dextrose vacutainers (Becton Dickinson, Franklin Lakes, NJ). DNA was extracted by a standard phenol-chloroform extraction procedure.[22] In brief, it consisted of the lysis of white blood cells, followed by protein digestion, extraction of the DNA with the help of phenol-chloroform, and precipitation of DNA with isopropanol.

4.2.4. Exome sequencing

Probands of each family were selected for exome sequencing, which was performed on a 5500XL sequencing platform from Life Technologies (Carlsbad, CA). The exomes of the probands were enriched according to the manufacturer's protocol using SureSelect^{XT} Human All Exon v.2 Kit (50Mb), containing the exonic sequences of approximately 21,000 genes from Agilent Technologies, Inc. (Santa Clara, CA). LifeScope software v2.1 from Life Technologies (Carlsbad, CA) was used to map color space reads along the hg19 reference genome assembly. The DiBayes algorithm, with high-stringency calling, was used for single-nucleotide variant calling. The small Indel Tool was used to detect small insertions and deletions. Exome sequencing data were filtered as described previously.[23,24] Briefly, the variants in known BBS genes were selected and analyzed for segregation in the families.

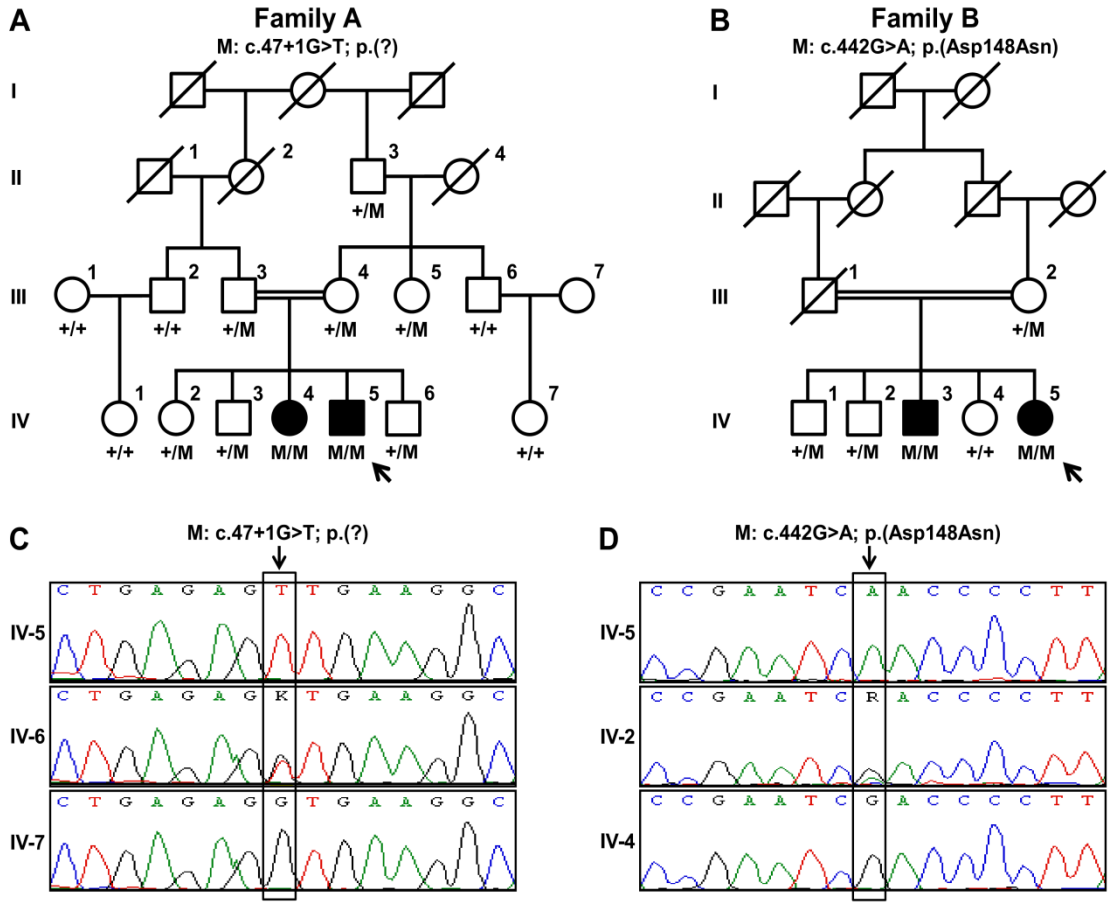

Figure 4.1 Pedigrees of families A and B and sequence electropherograms. A: Segregation of the identified mutation in family A. **B:** Segregation of the identified mutation in family B. **C:** Sequence electropherograms of an affected individual (upper panel), a heterozygous mutation carrier (middle panel) and a homozygous wild-type healthy individual (lower panel) of family A. **D:** Sequence electropherograms of an affected person (upper panel), a heterozygous mutation carrier (middle panel) and a homozygous wild-type healthy individual (lower panel) of family B. In family pedigrees, roman numerals indicate generation number, arrows indicate probands, M stands for mutation identified, + is wild-type allele, M/M indicates genotypes of affected individuals, +/M indicates genotypes of unaffected individuals carrying a mutant allele and +/+ indicates genotypes of healthy individuals.

4.2.5. Sequence analysis

Variants identified by exome sequencing were confirmed with Sanger sequencing using an automated DNA sequencing machine (3730 DNA analyzer; Applied Biosystems, Inc., Foster City, CA). PCR primers were designed with the help of the online tool Primer3.[25]

4.2.6. Splice site prediction

NetGene2 World Wide Web Server (http://www.cbs.dtu.dk/services/NetGene2/),[26,27] an online splice site prediction program, was used to predict the effect of sequence variants located in or near the splice sites.

4.2.7. In silico pathogenicity assessment of missense variant

Pathogenicity of the missense variant was assessed using the online prediction tools Polymorphism Phenotyping v-2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/) and Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html). In addition, HOPE (http://www.cmbi.ru.nl/hope/home) server was used to predict structural consequences in the mutant protein using normal protein structure with accession number Q8NFJ9.[28]

4.3. Results

4.3.1. Clinical findings

Affected individuals underwent extensive clinical examination, including fundus examination, which revealed the presence of bone spicules and attenuation of blood vessels (Figure 4.2). ERG measurements were recorded for affected individual IV:5 of family A only, which showed reduced scotopic and photopic electrophysiological responses in the patient as compared to the normal individual (Table 4.1). All other primary and secondary BBS features were also investigated (Table 4.2). Retinitis pigmentosa (RP), obesity and learning difficulties were present in all the affected persons while polydactyly was present in the affected female (IV:4) of family A and the affected persons IV:3 and IV:5 of family B. Developmental delay, a secondary feature of BBS was also observed in both families. Echocardiography was normal in both affected individuals of family A whereas in family B echocardiography was not performed, but affected individuals of family B were found to be hypertensive. Liver function was also normal and no renal defects were observed in both families.

Affected individuals of family B also had intellectual disability and dental crowding. All the patients fulfilled the diagnostic criteria of BBS having at least four primary, or three primary and two secondary BBS features.

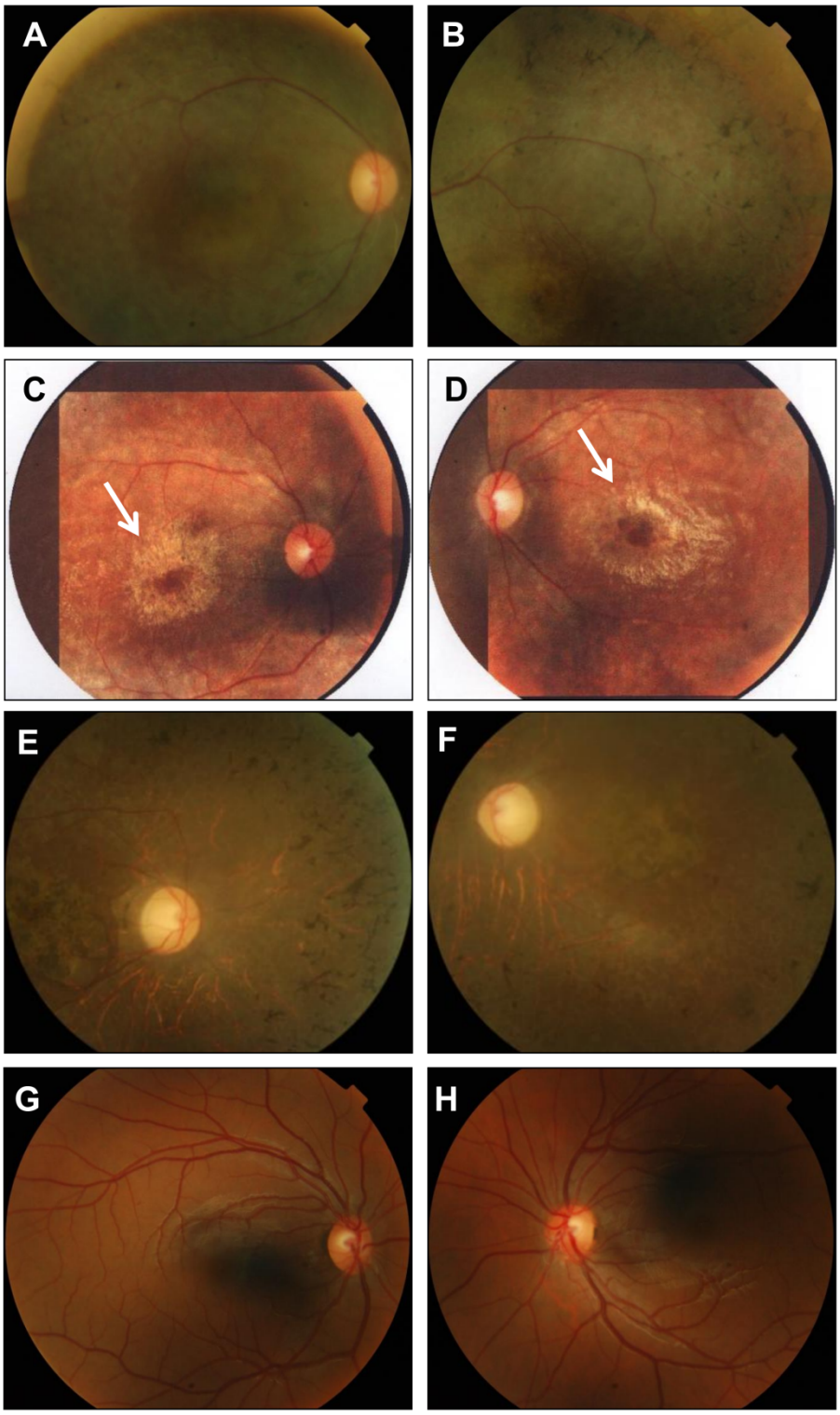

Figure 4.2 Fundus photographs of affected and healthy individuals. A, B: Fundus photographs of family A, proband IV:5, showing bone spicules, retinal vessel attenuation and macular degeneration. **C, D:** Fundus photographs of the proband's sister (IV:4) showing salt and pepper appearance of both fundi, presence of bone spicules in the mid periphery and bull's eye macular atrophy (indicated by the arrows). **E, F:** Fundus photograph of family B proband IV:5 revealing pigmentary deposits and retinal vessel attenuation. **G, H:** Fundus photographs of a healthy individual from family A (IV:6).

Table 4.1 Electrophysiological measurements recorded for individual IV:5 of family A.

Measured parameters using monopolar electrodes	Adaptation	Flash strength (cd.s/m^2)	Proband*	Control	Standard Values (Age=30 years)
Scotopic 25 dB b-wave amplitude (μV)	Dark	0.01	9.5	177.5	>163
Scotopic 0 dB b-wave amplitude (μV)	Dark	3.0	7.1	434.4	>403
Oscillatory potential amplitude (μV)	Dark	3.0	30.2	193.1	>89
Photopic 0 dB b-wave amplitude (μV)	Light	3.0	7.6	123.6	>92
Photopic 30 Hz flicker amplitude (μV)	Light	3.0	3.2	53.3	>63

*Proband's age was 26 at the time of the measurements.

4.3.2. Genetic findings

In order to identify the pathogenic mutation, the exome data sequence variants were filtered to reduce the number of potentially pathogenic variants. First, we searched for variants present in known BBS genes. In both families variants were found in six BBS genes, including *BBS1* (NM_024649.4), *BBS2* (NM_031885.3), *BBS4* (NM_033028.3), *BBS7* (NM_176824.2), *BBS9* (NM_198428.2) and *BBS12* (NM_001178007.1) (Table 4.3). The frequency of variants identified in known BBS genes except the *BBS1* variants described below, ranged from 13% to 99% in an in-house database.

In family A, a novel splice donor site mutation (c.47+1G>T) in *BBS1* was identified (Table 4.3). This mutation was present in a homozygous state in the exome data with only two reads, which was further confirmed as a homozygous change by Sanger sequencing (Figure 4.1C). Segregation analysis (Figure 4.1A) revealed that the mutation was homozygous in both affected siblings, heterozygous in parents and normal siblings and absent in some other unaffected members of the family. The c.47+1G>T variant is predicted to affect one of the canonical splice site nucleotides, which might therefore be completely inactivated. Splice site prediction software predicted inactivation of the wild type splice donor site at the 3' end of

exon 1 and did not predict an alternative splice donor site in intron 1. The mutant mRNA is likely to have a premature stop codon at position 17 (Figure 4.3).

Similarly in family B, exome data were analyzed for variants in known BBS genes which resulted in the identification of a previously reported missense mutation c.442G>A; p.(Asp148Asn) in *BBS1* (Figure 4.1D; Table 4.3). Segregation analysis (Figure 4.1B) revealed that this mutation was homozygous in the affected individuals (IV:3 and IV:5), heterozygous in the healthy siblings (IV:1 and IV:2) and their mother (III:2) and absent in one healthy sister (IV:4).

Table 4.2 BBS features in affected individuals of both families.

BBS features	**Present**		
Primary features	Family A IV:5	Family A IV:4	Family B IV:5
1. Rod-cone dystrophy	Yes	Yes	Yes
2. Polydactyly	No	Yes	Yes
3. Obesity	Yes	Yes	Yes
4. Learning problems	Yes	Yes	Yes
5. Hypogonadism	No	No	No
6. Renal malfunction	No	No	No
Secondary features			
1. Speech problems	No	No	Yes
2. Strabismus, cataract, astigmatism	Strabismus	Astigmatism	No
3. Brachydactyly, syndactyly	No	No	No
4. Developmental delay	Yes	Yes	No
5. Polyuria, polydipsia	No	No	No
6. Diabetes mellitus	No	No	No
7. Ataxia, imbalance	No	No	No
8. Mild spasticity	No	No	No
9. Dental crowding	No	No	Yes
10. Heart problems	No	No	Yes
11. Liver disease	No	No	No
12. Family members with BBS	Yes	Yes	Yes

Table 4.3 Variants identified in genes previously implicated in BBS through exome sequencing of families A and B

Family A

Chr/Position	Reads	Var reads	% var	SNP id	Freq	Gene	AA changes	mRNA changes	PhyloP	GS
11/66278178	2	2	100	-	-	*BBS1*	p.(?)	c.47+1G>T	3.03	-
16/56548501	109	106	97	rs4784677	97.09	*BBS2*	p.(Ser70Asn)	c.209C>T	2.50	46
16/56545175	119	46	39	rs11373	28.48	*BBS2*	p.(Ile123Val)	c.367T>C	0.23	29
15/73002035	45	44	98	rs8033604	76.70	*BBS4*	p.(?)	c.77-6G>A	-3.73	-
15/73023937	103	103	100	rs12914333	99.35	*BBS4*	p.(Phe302Phe)	c.906T>C	-0.58	-
15/73027478	20	20	100	rs2277598	78.64	*BBS4*	p.(Ile354Thr)	c.1061T>C	-0.39	89
4/122749541	68	22	32	rs1507994	12.94	*BBS7*	p.(?)	c.1890+16G>A	-0.92	-
7/33388713	87	27	31	rs11773504	35.92	*BBS9*	p.(Ala455Thr)	c.1363G>A	0.31	58
4/123664204	41	23	56	rs309370	51.46	*BBS12*	p.(Arg386Gln)	c.1157G>A	-0.34	43
4/123664919	99	62	63	rs13102440	36.57	*BBS12*	p.(Gln624Gln)	c.1872A>G	-0.05	-

Family B Chr/Position	Reads	Var reads	% var	SNP id	Freq	Gene	AA changes	mRNA changes	PhyloP	GS
11/66283020	51	51	100	-	-	*BBS1*	p.(Asp148Asn)	442G>A	3.54	23
16/56548501	92	92	100	rs4784677	97.02	*BBS2*	p.(Ser70Asn)	209C>T	2.50	46
15/73023937	73	72	99	rs12914333	99.19	*BBS4*	p.(Phe302Phe)	906T>C	-0.51	-
15/73027478	31	13	42	rs2277598	76.42	*BBS4*	p.(Ile354Thr)	1061T>C	-0.32	89
4/123664204	49	47	96	rs309370	52.85	*BBS12*	p.(Arg386Gln)	1157G>A	-0.32	43
4/123664427	50	15	30	rs13135766	29.27	*BBS12*	p.(Val460Val)	1380G>C	1.66	-
4/123664457	62	28	45	rs13135445	34.96	*BBS12*	p.(Cys470Cys)	1410C>T	-0.39	-
4/123664919	84	36	43	rs13102440	36.86	*BBS12*	p.(Gln624Gln)	1872A>G	0.01	-

AA, amino acid; Chr, chromosome; Freq, frequency; GS, Grantham score; PhyloP, phylogenetic P-values; SNP id, single nucleotide polymorphism identifier; Var, variation

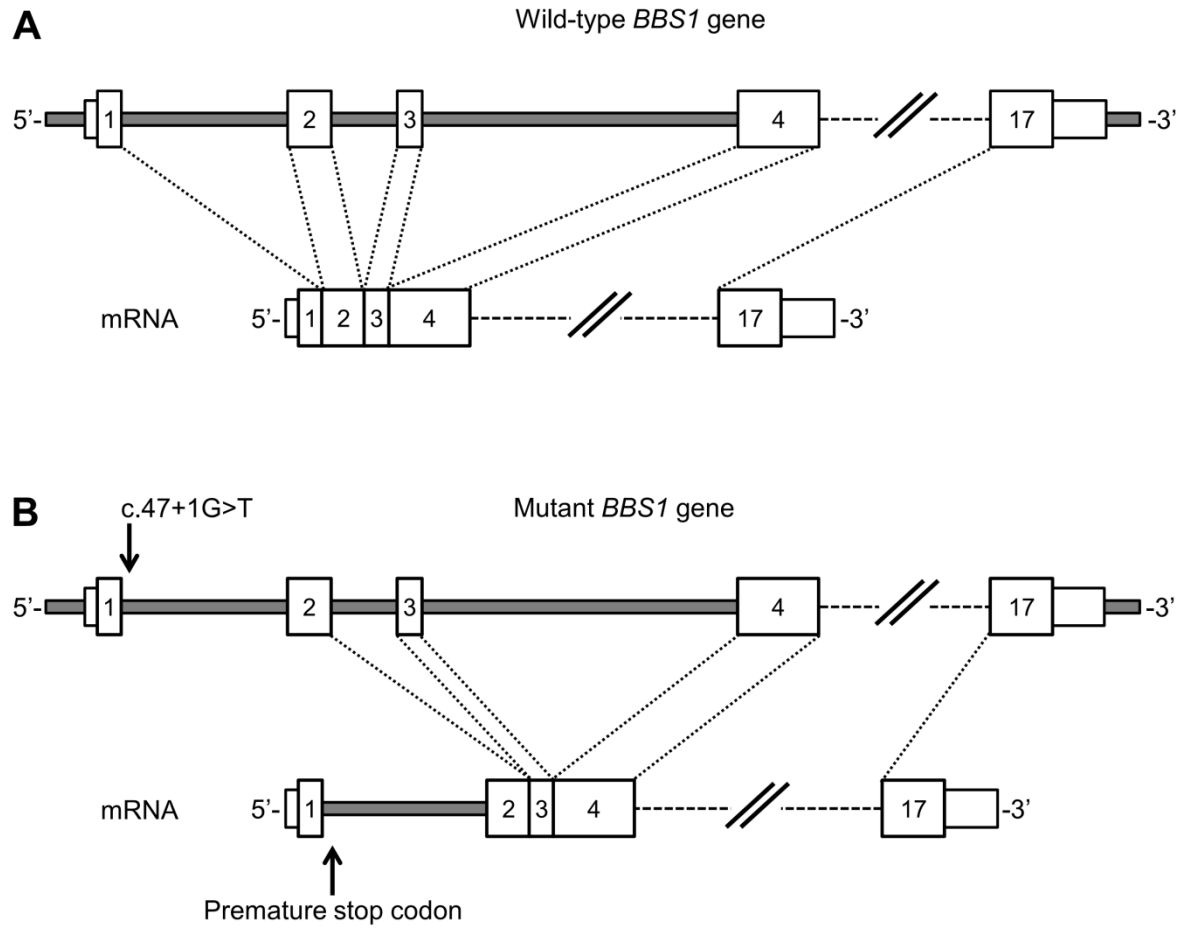

Figure 4.3 Predicted effect of splice donor site mutation c.47+1G>T on mutant *BBS1* mRNA splicing. A: Wild type *BBS1* and resulting mRNA after splicing are shown. **B:** Mutant *BBS1* and inclusion of intron 1 in the mutant mRNA in indicated which might result in the production of truncated BBS1 protein followed by non-sense mediated decay.

PolyPhen-2 predicted the missense mutation p.(Asp148Asn) as "probably damaging" whereas SIFT predicted this mutation to be a "tolerated" one. HOPE predicted that the mutation is present in the core of a domain; difference in the properties of wild type and mutant amino acid residues might disturb the core structure of this domain.

4.4. Discussion

In the current study we report on two consanguineous families from Pakistan, with affected individuals presenting BBS, which is a rare, clinically and genetically heterogeneous disorder. In approximately 75% of BBS families, mutations are detected in known BBS genes.[21] *BBS1* is the most frequently mutated BBS gene and accounts for the disease in nearly 24% of European patients with BBS.[4,29,30] The *BBS1* mutation p.(Met390Arg) is a frequent founder mutation[5,7,19] that is found in 78.3% of families with *BBS1* mutations.[5,21]

Including our families, more than 25 different Pakistani families have been described with variable BBS phenotypes and different mutations in *BBS2*, *BBS3*, *BBS4*, *BBS5*, *BBS8*, *BBS10*, and *BBS12*.[31-36] *BBS1* mutations have not been reported previously in Pakistani individuals with BBS.

The exact function of BBS1 in the pathology of BBS is still unclear but being a part of the BBSome complex, BBS1 is thought to play a crucial role by interacting with other proteins through its beta-propeller domain.[37] A detailed model of the assembly of the BBSome complex has been proposed recently,[38] which describes crucial steps required for the proper assembly of BBS proteins to form a functional BBSome complex. BBS1 joins the BBSome complex by interacting with BBS9 and BBS2, and the last component (BBS4) completes the BBSome complex assembly.[38] In the presence of $BBS1^{M390R/M390R}$ mutant protein, BBS4 fails to join the BBSome complex, which shows that protein-protein interactions between mutant BBS1 and wild-type BBS4 are lost.[38] The p.(Met390Arg) is a frequently occurring missense mutation in *BBS1* in persons with BBS that severely affects these normal protein-protein interactions.

The splice site mutation c.47+1G>T identified in the current study abolishes the splice site and is most likely to result in the synthesis of a truncated mutant protein as a consequence of intron inclusion. Truncation might result in nonsense-mediated decay of the mRNA because of the possible creation of a premature stop codon at position 17 (Figure 4.3); alternatively, usage of an alternate downstream translation initiation codon ATG might result in 5' truncation causing synthesis of misfolded/non-functional proteins. Misfolded proteins are mostly likely triggered towards the degradation pathways, which safeguard the cells from toxicity of the accumulating intermediary molecules.[39-41]

In family A, the main intra-familial phenotypic difference was the presence of polydactyly. In addition, bull's eye macular atrophy was observed only in the affected female, which also highlights the phenotype variability. Polydactyly is an observable clinical feature of BBS in almost all the BBS patients;[18] the absence of polydactyly in the proband of family A thus could be the effect of a still unknown modifier allele. A known modifier of ciliopathies (*RPGRIP1L*) has been functionally tested in zebrafish to observe modifying effect of different alleles.[9] In our family variants were not identified in *RPGRIP1L*. Moreover, although we did not test the functional effect of other identified variants on phenotype, their effect on phenotypic variability cannot be ruled out. Ophthalmological examinations revealed severely

reduced electrophysiological responses of rods and cones (Table 4.1), and the visual acuity was restricted to hand movements only, illustrating the severity of the disease. The proband of family B had intellectual disability and did not cooperate in conducting the electrophysiological measurements.

Contrary to our findings, in a recent study *BBS1* mutations were reported to be associated with milder ocular phenotypes as compared to the mutations in other BBS genes.[42] Moreover, in another study, milder non-ocular BBS phenotypes were reported in patients with mutations in *BBS12*.[35] In addition mutations in *BBS1*,[11] *BBS3*[43] and *BBS8*[44] are also implicated in non-syndromic retinitis pigmentosa instead of BBS.

In a pharmaco-genomic study aberrant splicing caused by a splice donor site mutation in *BBS1* (c.479G>A) was corrected in the patient's fibroblasts by using mutated U1 small nuclear RNA.[45] U1 snRNA is involved in the recognition of exons during splicing. The investigators generated mutant U1 small nuclear RNAs with increased complementarity for a mutated splice donor site, which were subsequently used to redirect correct splicing. Using a similar strategy, the splice donor site mutation (c.47+1G>T) identified in our study could first be assessed in *in vitro* studies and then in model organisms, which might then lead to the development of treatment options for individuals with this particular mutation in the future.

The missense mutation p.(Asp148Asn) was previously identified in an American and a British patient.[21] Since then this is the second report of this mutation. Although this mutation is distributed worldwide, it is rare in occurrence, being genetic cause of the disease in only four patients.

In the absence of a solved 3D structure or modeling template for the wild type BBS1 protein HOPE did not predict a 3D model for the mutant protein; but based on the differences in the amino acid properties it was predicted that wild type interactions of the protein might be disturbed due to the introduction of a mutant residue. Although *in silico* analysis is a good analytical tool to assess the pathogenicity of missense variants, functional validation is mandatory.

The use of exome sequencing to identify genetic mutations in BBS families was justified in our study as we compared the expenses of Sanger sequencing and exome sequencing. Expenditure of the Sanger sequencing (2140€) of the coding exons (228 exons; 214 amplicons) of 17 BBS genes in both directions is comparable with the exome sequencing

costs (1500-2000€). In addition Sanger sequencing requires longer hands-on time and more effort as compared to exome sequencing.

In conclusion, in Pakistani families with BBS, exome sequencing proved to be a successful and fast method for the identification of a novel as well as a recurrent mutation in *BBS1*. To our knowledge, this is the first report describing *BBS1* mutations in the Pakistani population. In the future, some of the clinical features might possibly be addressed using gene therapy, but currently, only genetic counseling is warranted for carriers of mutations.

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Chapter 5

Homozygosity mapping identifies genetic defects in four consanguineous families with retinal dystrophy from Pakistan

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ABSTRACT

Purpose: To identify the underlying genetic defects in four consanguineous retinal dystrophy families from Pakistan.

Method: Detailed clinical analysis that included funduscopy, electroretinography (ERG) and visual acuity (VA) assessment was done for at least one proband per family. Genome wide single nucleotide polymorphism (SNP) arrays were performed for multiple affected individuals in the respective families. The genotype data were analyzed to identify homozygous regions. Known autosomal recessive retinitis pigmentosa (arRP) and Leber congenital amaurosis (LCA) genes residing in the homozygous regions were screened for possible pathogenic variants.

Results: Clinical analysis showed variable signs of retinal degeneration, with and without macular involvement. Homozygosity mapping analysis yielded multiple homozygous regions per family. The largest or second largest homozygous region in all families contained known retinal dystrophy genes, which upon sequence analysis were found to contain pathogenic variants in four arRP/LCA genes. In three families previously identified protein truncating mutations were found in *ABCA4* (c.6658C>T; p.(Gln2220*)), *AIPL1* (c.834G>A; p.(Trp278*)), and *CERKL* (c.847C>T; p.(Arg283*)), whereas in one family a novel mutation c.1630C>T; p.(Arg544Trp) was found in *PDE6A*.

Conclusion: Consanguineous families are a rich resource to find disease causing variants by exploiting a homozygosity mapping approach. Four different genes were found to be mutated in four arRP families supporting previous observations, that retinal dystrophy is genetically heterogeneous in the Pakistani population.

5.1. Introduction

Retinitis pigmentosa (RP) is a progressive retinal degeneration in which the degeneration of rod photoreceptor cells precedes that of cone photoreceptor cells. Rod photoreceptor cells are responsive to dim light, and therefore affected individuals with RP at early stages experience night vision problems. As rod photoreceptor cells outnumber cones in the periphery, patients gradually lose their peripheral vision, a condition also known as tunnel vision. At later stages of the disease affected individuals completely lose their visual acuity as the neighboring cone photoreceptors also degenerate, leaving affected individuals handicapped for the rest of their life as currently no treatment is available for the majority of the cases.[1]

The fundus of persons with RP typically reveals bone spicule pigmentation prominently in the periphery, attenuation of retinal arterioles, and waxy pallor of optic disk. However, clinically the disease is very heterogeneous as most of these symptoms overlap with other related phenotypes like Leber congenital amaurosis (LCA) or cone rod dystrophy (CRD).[2,3] The gold standard for the differential diagnosis is electroretinography (ERG) which measures the electrical responses of the retina, and is able to differentiate between rod and cone-specific responses based upon retinal illumination by varying the intensity of light.[4] Genetic heterogeneity evidently explains the clinically heterogeneous nature of the disease as currently 56 genes are known to cause RP (http://www.sph.uth.tmc.edu/retnet/ January, 2012).

Thirty-four genes and three loci are known for autosomal recessive (ar) RP, and the search for novel disease genes continues as the known genes are thought to explain the disease in only about 50% of the patients.[5] Consanguineous families with multiple RP patients are a good resource to find novel genetic defects. In this paper; we analyzed four consanguineous Pakistani RP families, and identified pathogenic variants using the homozygosity mapping approach.

5.2. Methods

Four consanguineous families were recruited from different areas of Pakistan (Figure 5.1). The basic inclusion criteria were the presence of night blindness, bone spicule pigmentation, and tunnel vision. Patients were informed about the aims and objectives of the project before obtaining written consent. Detailed clinical analysis, which included visual acuity (VA) assessment, funduscopy, and ERG, was done for at least one proband with a phenotype that

was representative for all patients in the family.[4] Peripheral blood was drawn and was used for DNA extraction by the standard phenol-chloroform method.[6]

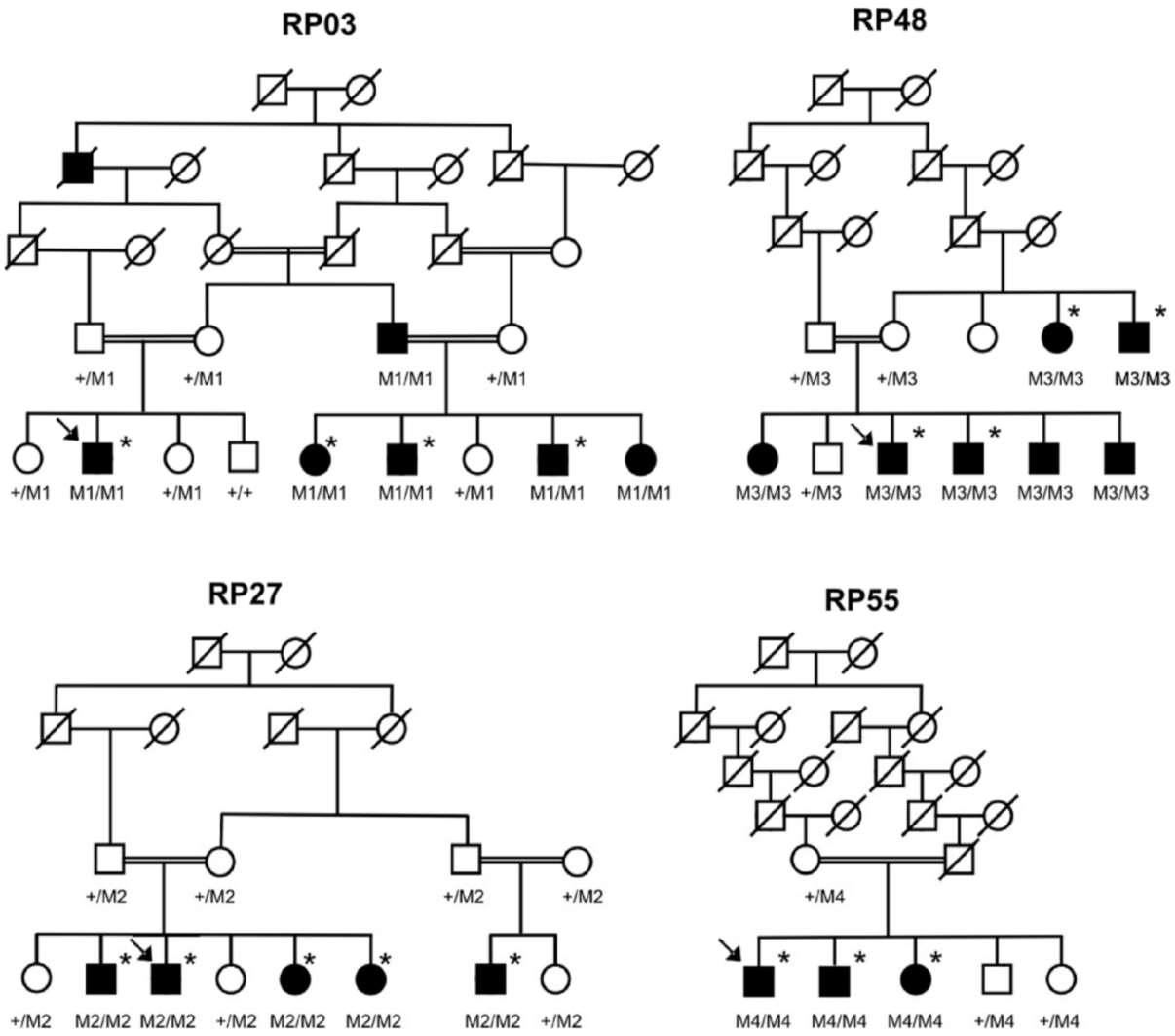

Figure 5.1 Segregation of genetic variants in families with autosomal recessive retinitis pigmentosa. Pedigrees of retinal dystrophy families and segregation analysis of pathological variants M1 (*ABCA4*; c.6658C>T; p.(Gln2220*)), M2 (*AIPL1*; c.834G>A, p.(Trp278*)), M3 (*CERKL*; c.847C>T, p.(Arg283*)), M4 (*PDE6A*; c.1630C>T, p.(Arg544Trp)). Individuals genotyped using whole genome single nucleotide polymorphism arrays are marked with asterisks. Arrows point to the probands.

Genomic DNA samples of selected probands, which are indicated as asterisks in the respective pedigrees were analyzed using whole genome SNP arrays (Figure 5.1). Families RP03 and RP27 were genotyped on Illumina 6K while RP48 and RP55 were analyzed on Human OmniExpress platform (700K). The genotyping data from families RP03, RP45, and RP55 were analyzed with the online mapping tool, Homozygosity Mapper.[7] For family RP27 linkage analysis was performed using the easyLINKAGE software package.[8] Thereafter the

haplotypes were compared among affected members of each family, and the regions in which all the affected individuals had the same SNP alleles were considered to harbor the pathogenic mutation.

Genomic coordinates of the known retinal dystrophy (RD) genes were obtained from the UCSC genome browser (http://genome.ucsc.edu) and were used to compare their location with the identified homozygous regions in each family. Sanger sequencing was performed to identify potential pathogenic variation in all the coding exons including exon-intron boundaries using primers designed with the online primer-designing tool ExonPrimer, primer sequences are available on request. Sequencing data were analyzed by Vector NTI, and the pathogenicity of the variants obtained were assessed based upon the functional consequences, nucleotide and amino acid conservation. In addition, the pathogenicity of a *PDE6A* missense variant was determined by publically available tools Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/), Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html) and Project Hope (http://www.cmbi.ru.nl/hope). To access the evolutionary conservation of the mutant residue, PDE6A amino acid sequences of different species were aligned which included humans (*Homo sapiens*, NM_000440.2); chimpanzees (*Pan troglodytes*, ENSPTRP00000029739); orangutans (*Pongo abelii*, ENSPPYP00000017834); macaque (*Macaca mulatta*, ENSMMUP00000006320), rat (Rattus norvegicus, ENSRNOP00000024171); mice (*Mus musculus*, ENSMUSP00000025468); dogs (*Canis familiaris*, ENSCAFP00000011716); platypus (*Ornithorhynchus anatinus*, ENSOANP00000014898); frogs (*Xenopus tropicalis*, ENSXETP00000027796); tetraodons (*Tetraodon nigroviridis*, ENSTNIP00000009080).

5.3. Results

5.3.1. Clinical data of the families

Families RP03, RP48, and RP55 show typical features of classic RP, as evidenced by the presence of attenuated retinal vessels, peripheral bone spicule pigmentation, and rod-dominated ERG abnormalities (Table 5.1; Figure 5.2). In proband of family RP48, a CRD pattern cannot be excluded as the macula was also affected at the time of examination (33 yrs). In family RP27, an early-onset form of RP was observed which is indistinguishable from LCA (Table 5.1).

5.3.1. Homozygosity mapping and sequence analysis

5.3.1.1. RP03

Family RP03 comprises of seven affected individuals one of which is deceased (Figure 5.1). Four out of the six available family members were genotyped (indicated by asterisks in the pedigree). Homozygosity Mapper analysis yielded eight homozygous regions (Table 5.2). The 2nd largest homozygous region harbored a known arRP gene, ATP-binding cassette, sub-family A member 4 (*ABCA4*; MIM #601691). Sequence analysis of all 50 protein coding exons revealed a homozygous nonsense mutation c.6658C>T; p.(Gln2220*) segregating with the disease in the family.

5.3.1.2. RP27

SNP microarray analysis of five affected individuals from two branches of family RP27 (Figure 5.1) yielded two homozygous regions (Table 5.2). The region on chromosome 17 was the largest and contained two known arLCA genes i.e. aryl hydrocarbon receptor interacting receptor protein like 1 (*AIPL1*; MIM #604392) and guanylate cyclase 2D (*GUCY2D*; MIM #600179). Sequencing of all 20 exons of *GUCY2D* revealed no variant. Sequence analysis of *AIPL1* exons revealed a nonsense substitution, c.834G>A; p.(Trp278*), that was segregating with the disease.

5.3.1.3. RP48

Analysis of genotyping data of four out of seven affected individuals (Figure 5.1) with Homozygosity Mapper indicated a single region on chromosome 2 (Table 5.2) that encompassed a known arRP gene, ceramide kinase-like (*CERKL*; MIM #608381). Upon sequence analysis of all 14 exons a nonsense substitution, c.847C>T; p.(Arg283*), was identified in exon 6 of *CERKL*, which was segregating in the family with the disease.

5.3.1.4. RP55

Homozygosity mapping with the genotype data from three affected individuals (Figure 5.1) revealed a single homozygous region on chromosome 5, which contains phosphodiesterase 6A (*PDE6A*; MIM #180071), a known arRP gene (Table 5.2). Sequence analysis of all 22 exons revealed a novel missense variant, c.1630C>T; p.(Arg544Trp), in exon 13. As shown in Figure 5.1, the mutation segregated perfectly in the family. The variant was considered to be

Table 5.1 Clinical features of affected probands with arRP/LCA

Family	Age at examination (yrs)	Age of onset (yrs)	Fundus	ERG	VA (OD, OS)	Diagnosis
RP03	30	10-15	ARV, Peripheral BSP	Rod-cone pattern	6/60, 6/60	RP
RP27	35	2-5	prominent macular degeneration,widespread BSP	Extinguished	LP	Early onset RP/ LCA
RP48	33	10-20	ARV, BSP, macular involvement	Rod-cone pattern	CF	RP
RP55	45	25-30	ARV, Peripheral BSP	Rod-cone pattern	6/60, 6/60	RP

ARV, attenuated retinal vessels; BSP, bone spicule pigmentation; CF, counting fingers; LP, light perception; ND, not determined; VA, visual acuity

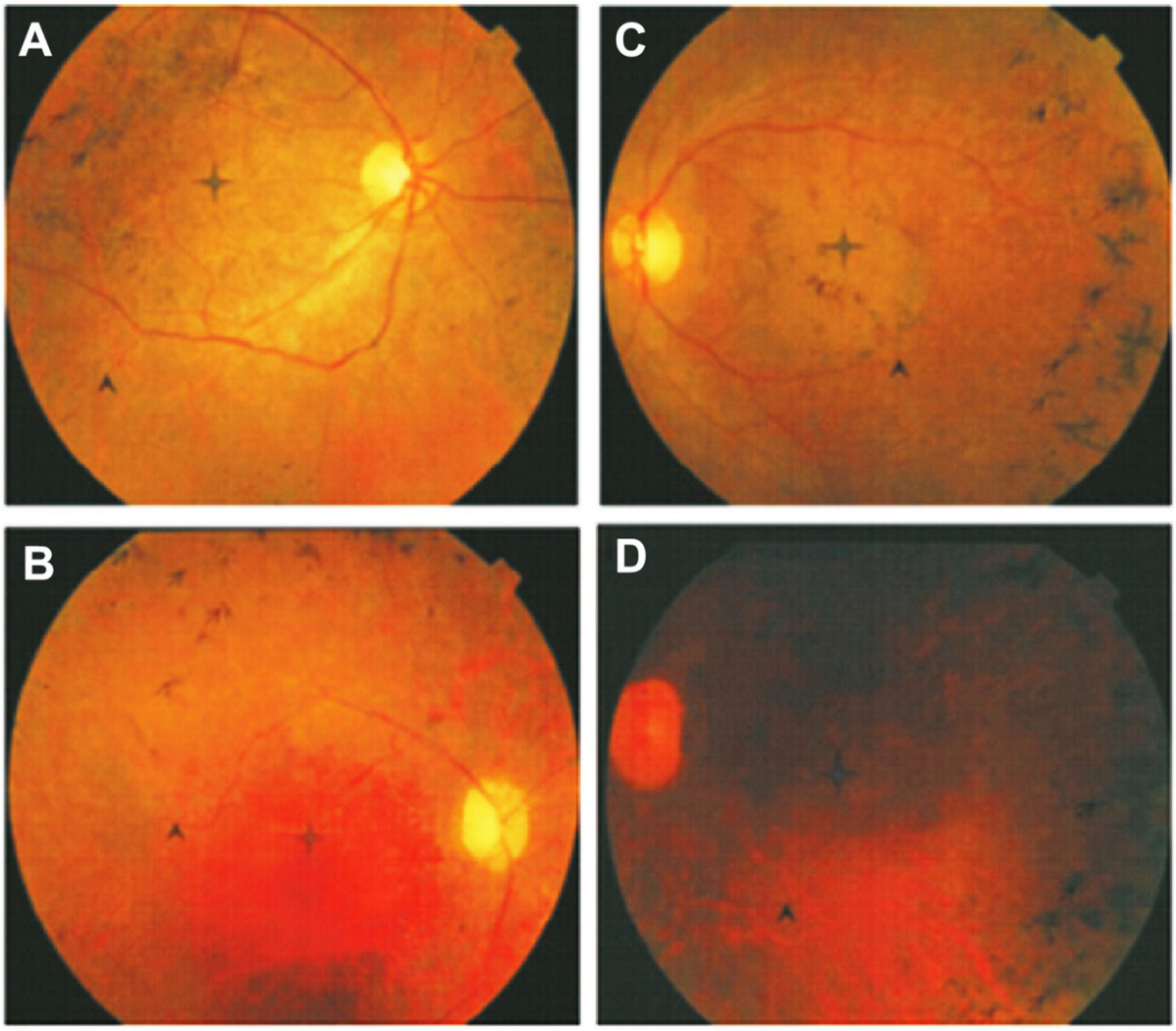

Figure 5.2 Fundus photographs of affected individuals from four families. Fundus photographs of proband RP03 (A), RP27 (B), RP48 (C), and RP55 (D). Bone spicule pigmentation at the retinal periphery (indicated by arrows), attenuation of retinal vasculature (indicated by arrow heads), and macular degeneration (indicated by stars) are evident in all cases.

likely pathogenic as it was not detected in 80 ethnically matched controls, the amino acid sequence conservation is very high (Figure 5.3), and it was predicted to be damaging and not tolerated by PolyPhen-2 and SIFT analysis, respectively.

5.4. Discussion

In this paper, four mutations in four different genes were described to be causative for arRP/LCA in the respective families. The *ABCA4* homozygous null mutation c.6658C>T; p.(Gln2220*), identified in arRP family RP03, was previously found in a heterozygous state in an isolated CRD patient.9 In that patient subsequent mutation analysis revealed a splice

Table 5.2 Homozygous regions found in arRP/LCA families

Family	Chr	Flanking SNP	Position (hg19)	Size in Mb	Ranking	Known arRP/LCA genes
RP03	1	rs743117;rs716581	94.4-102.3	7.9	2	*ABCA4*
	2	rs20349;rs17722726	134.6-137.6	3.0	7	----------
	4	rs730349;rs936232	21.2-25.8	4.6	5	----------
	4	rs543337;rs1461605	82.7-92.0	9.3	1	----------
	6	rs2894891;rs1475270	100.2-106.7	6.5	3	----------
	7	rs4728251;rs1424376	131.8-137.7	5.9	4	----------
	14	rs1007813;rs9324014	96.0-100.2	4.2	6	----------
	14	rs17172;rs872945	101.4-103.5	2.1	8	----------
RP27	17	rs884250;rs1860300	4.3-11.1	6.8	1	*AIPLI, GUCY2D*
	21	rs1012959;rs2837536	38.1-41.6	3.5	2	----------
RP48	2	rs6734093;rs12693396	182.2-185.7	3.5	1	*CERKL*
RP55	5	rs7711546;rs10515714	143.6-154.9	11.3	1	*PDE6A*

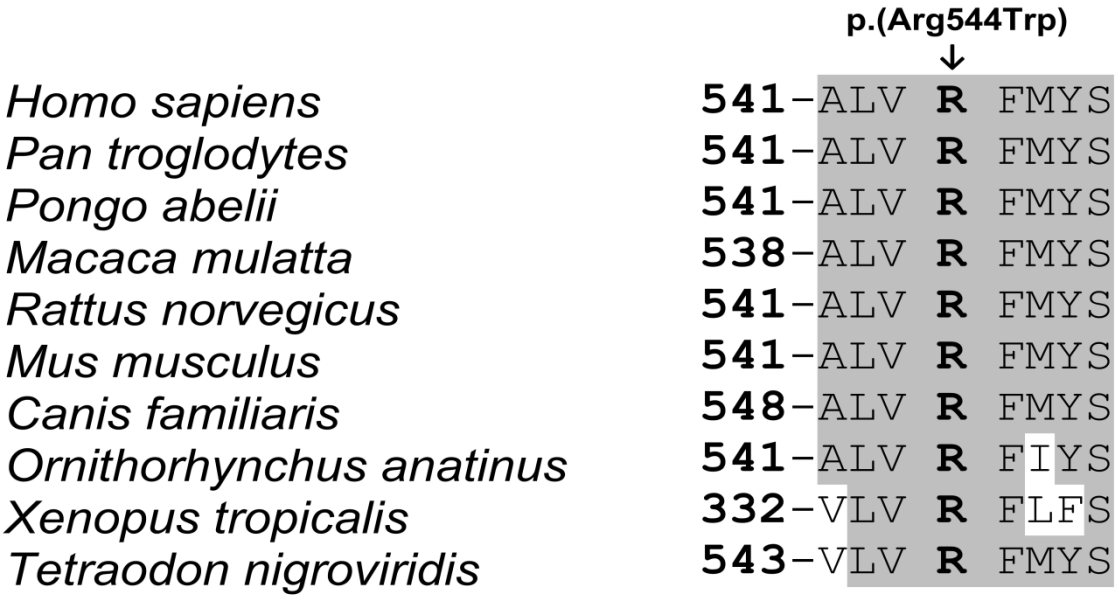

Figure 5.3 Amino acid conservation of arginine residue 544 in PDE6A that is mutated in family RP55. The amino acid position of the first residue in the respective protein is indicated.

defect c.4540-2A>G as the second allele (A. Maugeri and F.P.M.C, unpublished data). According to a genotype–phenotype model, two severe *ABCA4* mutations, such as a null mutation in RP03, causes a severe phenotype like RP, while a combination of a severe and a moderately severe mutation causes CRD, and a mild and a severe mutation cause Stargardt disease.[9-13] Thus far, pan-retinal cone-rod dystrophy or severe RP was exclusively associated with two severe *ABCA4* variants.[11,13,14] The p.(Gln2220*) variant is located in exon 48, which predicts that nonsense mediated decay of the RNA will occur and thus we consider this variant to be a null allele. This is in agreement with the observed phenotype in three clinically investigated affected individuals.

The *AIPL1* mutation c.834G>A; p.(Trp278*) that we found in family RP27 is the most common *AIPL1* mutation described worldwide.[15-17] This mutation was first described in a Pakistani family with LCA and more recently, the same mutation was found in three additional LCA families from Northern Pakistan.[15-17] The patients described in our study were diagnosed at a later stage (>30 years) of the disease, but due to the macular involvement, low visual acuity and non-detectable ERG of the proband, a LCA phenotype could not be ruled out.[16]

In family RP48 the *CERKL* mutation c.847C>T; p.(Arg283*) was found to be causative for arRP. This mutation was described for an isolated CRD patient of French-Canadian origin.[18] At the age of 33, the proband of family RP48 was diagnosed to be at an advanced stage of arRP, as the ERG suggested a rod-cone disease pattern. However, as we observed the macula and fundus abnormalities (Table 5.1) and as RP and CRD phenotypes tend to converge at late stages of disease, we cannot rule out CRD for this family.[18] A novel *PDE6A* variant c.1630C>T; p.(Arg544Trp) was identified in family RP55. The family was diagnosed with typical arRP, as the patients have prominent peripheral bone spicule pigmentation, arterial attenuation, and an intact macula (Table 5.1). PDE6A is part of a heterotetrameric complex, containing cyclic guanosine monophosphate-phosphodiesterase (cGMP-PDE), which is involved in mediating the phototransduction cascade as it forms the link between light mediated rhodopsin (RHO) activation and cGMP-gated ion channel closure, by hydrolyzing the cGMP upon activation.[19] Eighteen different mutations have been identified in *PDE6A*, the majority of which (56%; 10/18), are missense variants similar to the novel nucleotide change that we found in our family.[20-25]

At the protein level, the wild type arginine 544 is large, positively charged, and hydrophilic. This residue is replaced by a tryptophan in family RP5, which is even larger, neutrally charged, and hydrophobic. The wild type residue is present on the surface of the protein, close to the interaction interface with the other monomer (Figure 5.4A and B). As arginine is positively charged and hydrophilic, it can make important hydrogen bonds and ionic interactions with residues in the surrounding alpha-helices. These interactions probably stabilize the dimer interface necessary for the correct enzymatic function of the protein. Loss of these interactions due to the p.(Arg544Trp) mutations will destabilize the local structure, affect the interaction surface and hence affect the function of the protein.

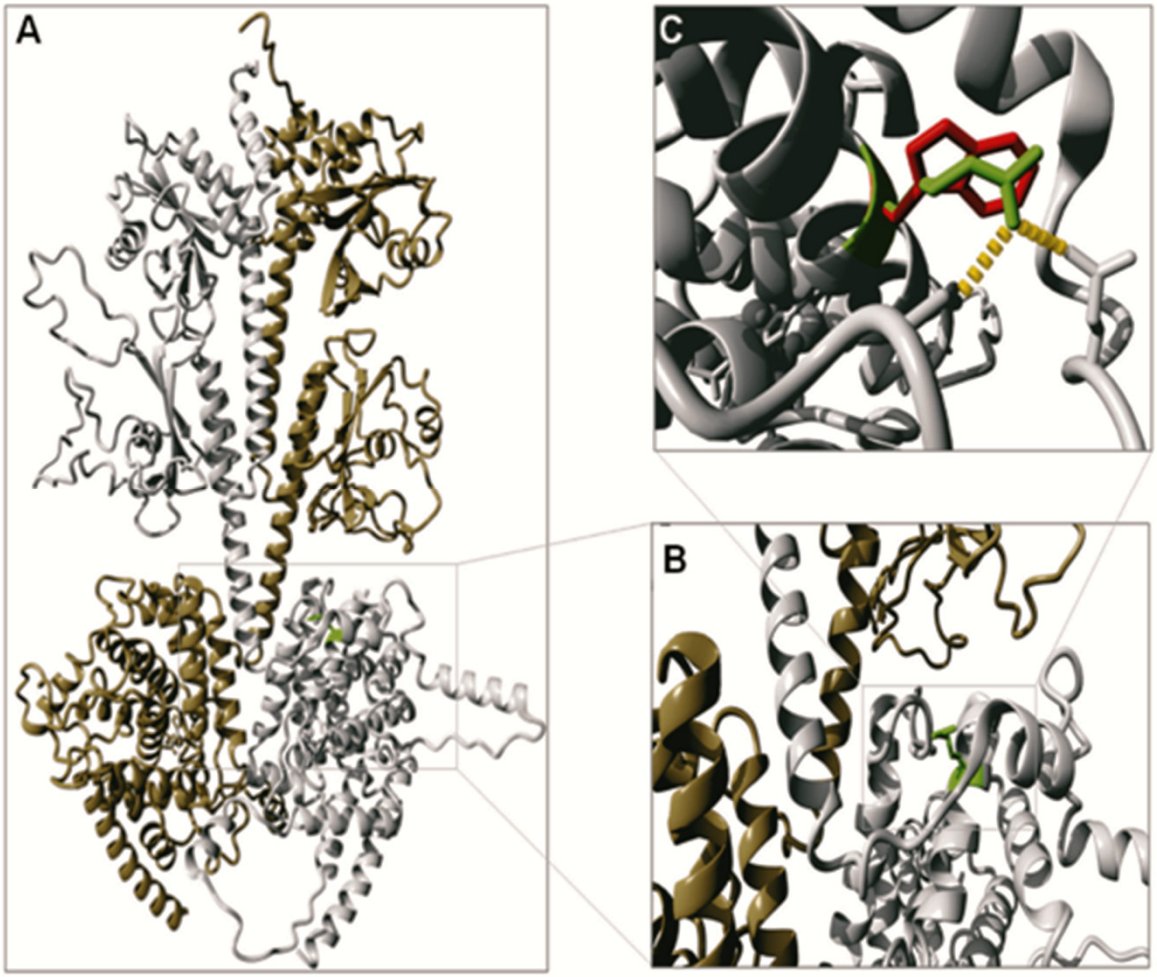

Figure 5.4 Protein modeling of PDE6A missense variant. Three-dimensional structure of heterotetrameric complex of cyclic guanosine monophosphate phosphodiesterase (cGMP-PDE). (A) Structure of PDE6A (gray) and PDE6B (tan) protein complex in the native conformation. Arg544 is colored green. (B) Part of PDE complex showing the location of the mutant residue, on the surface, close to the monomer interaction site. (C) Close-up of the mutation. Wild-type and mutant residues are shown and colored green and red, respectively. Yellow dots indicate the hydrogen bond formed by the wild-type residue, which will be lost due to the mutation.

In three families the gene with the pathogenic mutation was present in the largest homozygous region and in one family *RP03*, causative gene was present in the second largest homozygous region. This observation therefore points towards analyzing the highest ranked linkage interval first while searching for the causative variants in consanguineous families where several homozygous regions are commonly observed. Similarly, the same information can be used to filter variants obtained from next-generation sequencing if linkage data are available.

In conclusion, we have identified the genetic defects in four Pakistani families with retinal dystrophy by homozygosity mapping and sequence analysis of *ABCA4*, *AIPL1, CERKL,* and *PDE6A*. The present study will improve genetic diagnosis for other genetically uncharacterized patients. The identification of genetic defects will not only be helpful in counseling of affected individuals but will also be beneficial in selecting patients who are eligible for gene therapy in the near future.

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Chapter 6

Genetic causes of retinal dystrophies in Pakistan

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Manuscript in preparation

ABSTRACT

Homozygosity mapping has been successful in mapping autosomal recessive inherited disease loci. The customary consanguineous nuptials in Pakistan underlies the frequent occurrence of autosomal recessive (ar) inherited disorders, including retinal diseases (RD). Pakistani families with arRD have been instrumental in finding ten RD-associated genes and have enabled the recognition of novel genotype-phenotype correlations for three genes. By employing homozygosity mapping, we report on the identification of the causal genetic defects in 8 families with arRD. In another 9 families, we excluded 8 genes previously implicated in ar retinitis pigmentosa and Leber congenital amaurosis that were located in significant homozygous regions. For most families more than one homozygous region was identified with LOD scores varying between 1.8 and 4.0. Taking into consideration previously published studies, we could genetically solve 32 of 41 (78%) families, suggesting that the majority of genes underlying inherited non-syndromic RD have been identified. Ninety percent of mutations causing non-syndromic RD and all mutations causing syndromic forms in Pakistani families have not been reported in other human populations. Based on our inventory of all Pakistani RD-associated gene defects, we propose a cost-efficient allele-specific analysis of 13 RD-associated variants that may capture up to 42% of the genetic causes.

6.1. Introduction

Inherited retinal dystrophies (RD) belong to clinically and genetically heterogeneous disorders.[1] The clinical sub-classification of these diseases is based on the nature of the disease (stationary or progressive), the inheritance pattern, and the dysfunctional part of the retina.[2] The disease is either congenital, occurring early in life like Leber congenital amaurosis (LCA; MIM# 204000), or congenital stationary night blindness (CSNB; MIM# 310500), or might have a later onset such as in retinitis pigmentosa (RP; MIM# 268000), cone-rod dystrophy (CRD; MIM# 604116), and cone dystrophy (CD; MIM# 602093).[3] In addition to disorders that are confined to the eye, there are syndromic forms of the disease in which blindness due to retinal cell degeneration is either among the primary clinical symptoms or might be manifested at advanced stages. The most common syndromic form of RD is Usher syndrome (USH; MIM# 276900), in which RP is coupled with variable degrees of hearing loss and vestibular dysfunction.[4] Other types of syndromic RD include Bardet-Biedl syndrome (BBS; MIM# 209900), Senior-Loken syndrome (SLSN; MIM# 266900), Joubert syndrome (JBTS; MIM# 213300), and Meckel syndrome (MKS; MIM# 249000), all of which exhibit severe overlapping clinical features in addition to retinal degeneration.[5,6]

The estimated worldwide prevalence of RD is 1 in 3,000 individuals.[7] RP is the most frequent among these diseases, affecting 1 in 4,000 individuals.[8,9] In Pakistan the frequency of RD is not well defined but a hospital-based study estimated it to be 1 in 800 patients, with autosomal recessive RP being the most prevalent.[10] This is because more than 60% of marriages in the country are consanguineous and among them about 80% are between first cousins.[11] In several developing countries, as opposed to the Western countries, consanguinity has always been a major contributing factor in the high prevalence of autosomal recessive disorders.[12] Such consanguineous families are ideal for homozygosity based genetic mapping studies aimed at the identification of the underlying genetic defect.[13,14]

Facilitated by several technological advances, to date 185 genes implicated in different inherited RD have been identified (http://www.sph.uth.tmc.edu/retnet/sum-dis.htm). Among these genes, 119 are linked to non-syndromic forms of the disease with genetic overlap between different classes.[1,3,15] In the developed countries genetic testing using medium-to-high throughput genotyping methods is now being used for proper disease diagnosis.[16] This has resulted in the establishment of many genotype-phenotype correlations.[16-18] In the last two decades, several studies have described the genetic causes of different retinal dystrophies in

consanguineous Pakistani families. However, there is no comprehensive overview of all forms of RD that have been studied in Pakistan. In this study we describe homozygosity mapping and sequence analysis results for 17 Pakistani RD families, which are part of a cohort of 41 randomly collected Pakistani RD families. In addition, we provide an overview of all published genetic data of syndromic and non-syndromic retinal dystrophies that have been described for Pakistani families.

6.2. Methods

6.2.1. Genetic analyses of 41 RD families

This study was dually approved by the institutional Ethics Committee and was conducted in accordance to Helsinki declaration. A panel of 41 retinal dystrophy families, including 38 non-syndromic and 3 syndromic families (i.e., two BBS and one SNLS), were selected for single nucleotide polymorphism (SNP) microarray based genotyping. The non-syndromic cohort consisted of families with autosomal recessively inherited RP (n=31), CSNB (n=4), CD (n=2), and a family with X-linked RP, details of the respective families are presented in Table 6.1. Preliminary analysis that included patient recruitment, sample collection and DNA isolation have been described previously.[19,20] Novel genetic data of 17 families are presented here. Twenty-two families from this cohort were published elsewhere.[19-28] Genetic mapping of the panel of 41 families was carried out using single nucleotide polymorphism (SNP) microarrays from Illumina (10K), Affymetrix (6K), or HumanOmniExpress (700K). As most of the families were consanguineous and pedigree analysis suggested an autosomal recessive mode of disease inheritance, the homozygosity mapping approach was utilized to find the causal gene. The genotype data were analyzed using an online Homozygosity Mapper tool.[29] Subsequently, using Gene Hunter in the easyLINKAGE program, parametric linkage analysis was performed to calculate the LOD scores for the homozygous regions.[30] Haplotypes were compared and only the regions that were identical in all the affected individuals of each family were considered further. Whenever available, genotype data from healthy individuals were used to exclude the regions that were found to be homozygous in both healthy and affected individuals. In case no unambiguous homozygous region was identified in families with multiple sibships, homozygosity mapping was performed for separate branches of the family. After haplotype analysis, regions were prioritized based upon size and/or the presence of genes known to be implicated in inherited RDs. Sequencing primers were designed from the UCSC genome browser (http://genome.ucsc.edu), using ExonPrimer. Sanger sequencing

Table 6.1 Overview of retinal disease phenotypes and affected families

Phenotypes	Clinical characteristics	Families analyzed	Affected individuals	Solved Families	Solved patients
RP early/late onset	Peripheral vision loss, night blindness, bone spicule pigmentary deposits, reduced rod response	32	187	23‡	144
CSNB (Oguchi, FA)	Congenital stationary night blindness, whitish dots in fundus (FA), Mizuo phenomenon (Oguchi)	4	19	4	19
CD and ACHM	Reduced central vision, poor color vision, photophobia, nystagmus, bull's eye macula	2	8	2	8
BBS	Retinal dystrophy, polydactyly, obesity, intellectual disability	2	4	2	4
SLSN	Early onset RP, end stage renal failure in first decade of life	1	3	1†	3
Total		41	221	32	178

ACHM, achromatopsia; BBS, Bardet-Biedl syndrome; CD, cone dystrophy; CSNB, congenital stationary night blindness; FA, fundus albipunctatus; RP, retinitis pigmentosa; SLSN, Senior-Loken syndrome. ‡7 of these families are part of this study, †part of this study.

was carried out to screen exons and flanking intronic sequences of all RD genes residing in the homozygous regions in one proband from each family. Segregation analysis of identified mutations was also done using Sanger sequencing. Sequence variants were characterized based upon nucleotide conservation (PhyloP score), a bioinformatics prediction of the biological consequences of amino acid substitutions was performed using Polymorphism Phenotyping v2 (Polyphen-2) (http://genetics.bwh.harvard.edu/pph2/), and Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html), and whether the change affects an amino acid in a functional domain of the corresponding protein. Novel variants were also checked in population-matched healthy controls by restriction fragment length polymorphism (RFLP) analysis and/or Sanger sequencing. The exome variant server (EVS; a database containing variants retrieved from the National heart, lung and blood institute exome sequencing projects), and an in-house exome database were also checked for the presence of putative pathogenic variants.

6.2.2. Literature review

A comprehensive literature review was performed for mutations and loci which have been described previously for Pakistani individuals with syndromic and non-syndromic retinal diseases. The Retinal Network (RetNet; https://sph.uth.tmc.edu/retnet/disease.htm), National Centre for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov/), Online Mendelian Inheritance in Man (OMIM; http://www.ncbi.nlm.nih.gov/pubmed/), and The Human Gene Mutation Database (HGMD; http://www.biobase-international.com-/product/hgmd) were used to search for information and published literature.

6.3. Results

6.3.1. Homozygosity mapping and implication of previously identified causative genes in 8 retinal disease families

In 7 families with non-syndromic arRD (F01-F07), and one family (F08), with SLSN (Figure 6.1), homozygosity mapping not only revealed outstanding homozygous regions encompassing genes previously implicated in the respective phenotypes, but also enabled us to identify the causative mutations (Table 6.2). In two families novel mutations were identified in *CNGA1* (MIM# 123825: c.1298G>A; p.(Gly433Asp) in F01), and *CNGB1* (MIM# 600724: c.2493-2A>G; p.(?) in F04). In five other unrelated non-syndromic Pakistani RP families, previously reported mutations were identified (Table 6.3 and 6.4). These include

two mutations in *RPE65* (MIM# 180069: c.131G>A; p.(Arg44Gln) in F05 and c.361del; p.(Ser121Leufs*6) in F02), one mutation in *CRB1* (MIM# 604210: c.2234C>T; p.(Thr745Met) in F06), and one variant in *TULP1* (MIM# 602280: c.1466A>G; p.(Lys489Arg) in F07) (Figure 6.1, Table 6.3). In a family with nine affected individuals in four generations (F03; Figure 6.1), X-linked inheritance was plausible. In this family sequence analysis of *RPGR* (MIM# 312610), revealed a previously identified mutation (c.2426_2427del; p.(Glu809Glyfs*25); Table 6.3).

Finally, in family F08, with SLSN, a mutation affecting one of the canonical nucleotides of a splice acceptor site (c.488-1G>A; p.(?)) was identified in *IQCB1* (MIM# 609237), (Table 6.4). Mutations in *IQCB1* are the most frequent cause of SLSN, and the mutation we identified in family F08 has also been previously reported in another Pakistani SLSN family.[31]

6.3.2. Homozygosity mapping and exclusion of previously identified causative genes in 9 retinal disease families

Homozygosity mapping in three families (F09, F11, and F16), did not yield homozygous regions >2 Mb encompassing genes previously implicated in arRP or arLCA (Figure 6.2; Table 6.5). In the remaining 6 families, we excluded 8 arLCA or arRP genes located in homozygous regions (Figure 6.2; Table 6.5).

Among these 9 families homozygous regions in two families reached significant LOD scores (>3.3) on chromosome 5 (F12), and chromosomes 11 and 10 (F09), respectively, while in two families (F10 and F16), LOD scores of 2.7 and 3.0 were observed, which is highly suggestive of linkage at chromosomes 1 (F10), and chromosomes 4 and 17 (F16). In the remaining five families (Figure 2), the limited number of affected individuals that were tested restricted the maximally achievable LOD score. Details about the homozygous regions of the respective families are presented in Table 6.5.

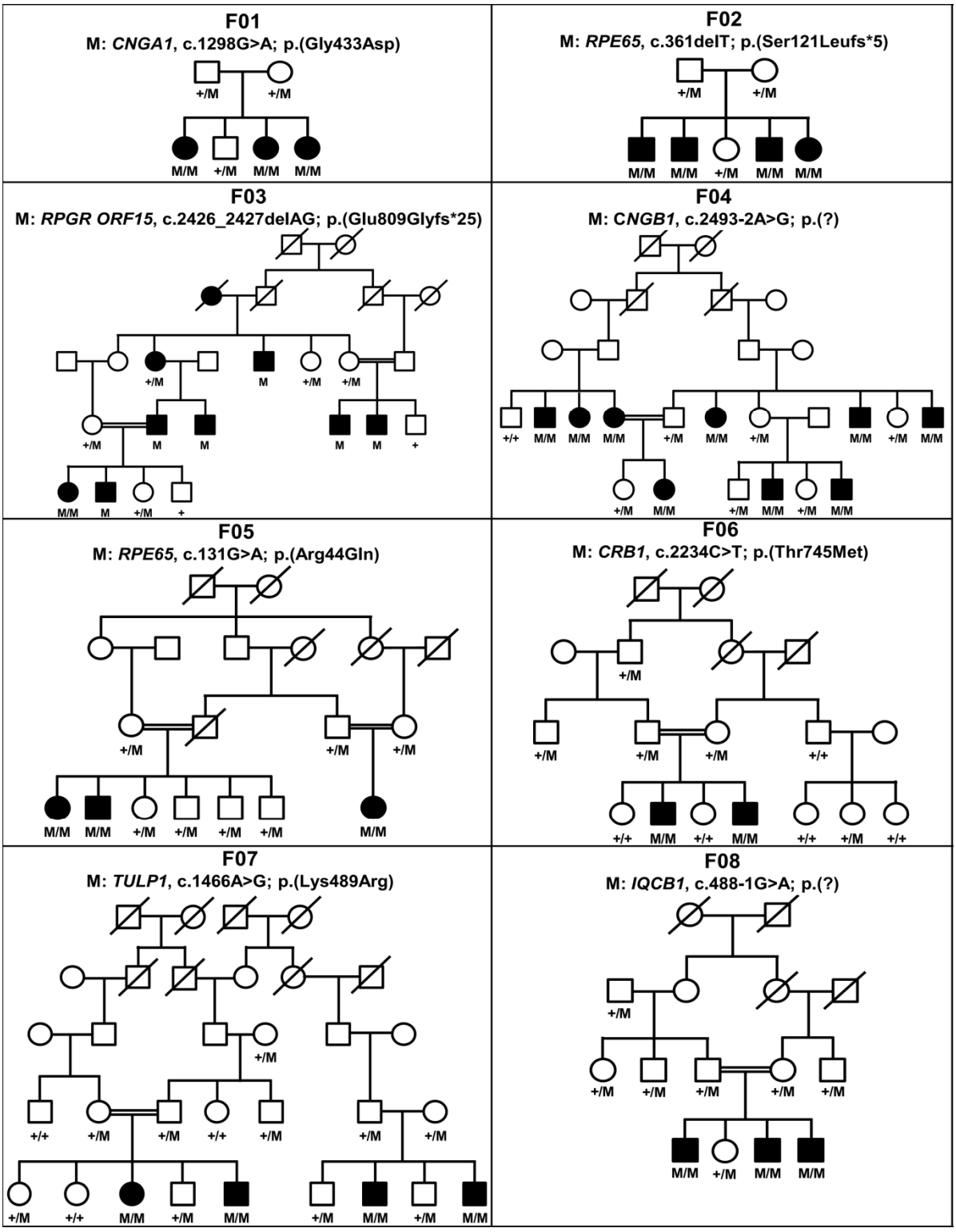

Figure 6.1 Pedigrees of families with inherited retinal diseases and segregation analysis of mutations. In Family 03 an X-linked mutation segregates; in the other families the inherited pattern is autosomal recessive. Black symbols represent affected individuals; open symbols non-affected persons. M stands for mutation identified, + is wild-type allele, M/M indicates affected genotypes, +/M indicates carriers of the mutant allele, and +/+ indicates genotypes of normal individuals.

Table 6.2 General information of the families describe in this study

Family	affected	Homozygous regions (*n*)>2 Mb	Size of homozygous region (Mb)	Ranking of region	arRP/LCA gene in region	Mutation identified	Mutation
F01	3	4	18.8	1	*CNGA1*	Yes	c.1298G>A; p.(Gly433Asp)
F02	4	2	5.2	1	*RPE65*	Yes	c.361del; p.(Ser121Leufs*6)
F03	9	NA	NA	NA	*RPGR*	Yes	c.2426_2427del; p.(Glu809Glyfs*25)
F04	9	1	6.5	1	*CNGB1*	Yes	c.2493-2A>G; p.(?)
F05	3	2	1.6	2	*RPE65*	Yes	c.131G>A; p.(Arg44Gln)
F06	2	5	13.4	2	*CRB1*	Yes	c.2234C>T; p.(Thr745Met)
			4.5	3	*LRAT*	No	NA
			3.3	4	*GUCY2D*	No	NA
F07	4	4	9.2	1	*TULP1*	Yes	c.1466A>G; p.(Lys489Arg)
F08	3	15	30.3	1	*IQCB1*	Yes	c.488-1G>A; p.(?)
			30.3	1	*RHO*	No	NA

LCA, Leber congenital amaurosis; Mb, megabases; NA, not applicable; RP, retinitis pigmentosa.

Table 6.3 Mutations identified in Pakistani patients with non-syndromic retinal dystrophies

Gene	Nucleotide variant	Protein variant	Phenotype	# of families	# of patients	References
ABCA4	c.6658C>T	p.(Gln2220*)	arRP	1	5	Maugeri, *et al*.,[36] Khan *et al*.[28]
AIPL1‡	c.834G>A	p.(Trp278*)	arEORP	11	25	Damji, *et al*.,[37] McKibbin, *et al*.,[38] Sohocki, *et al*.,[39] Khan *et al*.[28]
BEST1‡	c.418C>G	p.(Leu140Val)	adRP	1	4	Davidson, *et al*.[33]
CERKL	c.316C>A	p.(Arg106Ser)	arRP	1	3	Ali, *et al*.[40]
CERKL	c.847C>T	p.(Arg283*)	arRP	1	6	Avila-Fernandez, *et al*.,[41] Littink, *et al*.,[42] Khan *et al*.[28]
CLRN1†	c.92C>T	p.(Pro31Leu)	arRP	1	6	Khan, *et al*.[27]
CLRN1†	c.461T>G	p.(Leu154Trp)	arRP	1	6	Khan, *et al*.[27]
CNGA1	c.626_627del	p.(Ile209Serfs*26)	arRP	1	7	Zhang, *et al*.[43]
CNGA1	**c.1298G>A**	**P.(Gly433Asp)**	**arRP**	**1**	**3**	**This study**
CNGA3	c.822G>T	p.(Arg274Ser)	arCRD (ACHM)	1	4	Azam, *et al*.[24]
CNGA3	c.827A>G	p.(Asn276Ser)	arCRD (ACHM)	1	6	Saqib, *et al*.[44]
CNGB1	c.412-1G>A	p.(?)	arRP	1	10	Azam, *et al*.[25]
CNGB1	c.2284C>T	p.(Arg762Cys)	arRP	1	5	Azam, *et al*.[25]
CNGB1	**c.2493-2A>G**	**p.(?)**	**arRP**	**1**	**10**	**This Study**
CNGB3	c.1825del	p.(Val609Trpfs*9)	arCRD (ACHM)	1	2	Azam, *et al*.[24]
CRB1	c.107C>G	p.(Ser36*)	arLCA	1	10	McKibbin, *et al*.[38]

CRB1	c.116C>A	p.(Thr39Asp)	arLCA	1	6	Khaliq, *et al*.[45]
CRB1	**c.2234C>T**	**p.(Thr745Met)**	**arRP**	**1**	**2**	den Hollander, *et al*.,[46] **this study**
CRB1	c.2536G>A	p.(Gly846Arg)	arRP	1	6	Khaliq, *et al*.[45]
CRB1	c.3101T>C	p.(Leu989Thr)	arLCA	1	8	Khaliq, *et al*.[45]
CRB1	c.3296C>A	p.(Thr1099Lys)	arRP	1	9	Azam, *et al*.[25]
CRB1	c.3343_3352del	p.(Gly1115Ilefs*23)	arRP	1	9	Lotery, *et al*.[47]
CRB1	c.3347T>C	p.(Leu1071Pro)	arRP	1	7	Khaliq, *et al*.[45]
CRB1	c.3962G>C	p.(Cys1321Ser)	arRP	1	5	Lotery, *et al*.[47]
EYS	c.8299G>T	p.(Asp2767Tyr)	arRP	1	7	Khan, *et al*.[20]
GNAT1	c.386A>G	p.(Asp129Gly)	arCSNB	1	1	Naeem, *et al*.[48]
GRK1	c.614C>A	p.(Ser205*)	arCSNB (Oguchi)	1	9	Azam, *et al*.[19]
GRK1	c.827+623_883del	p.(?)	arCSNB (Oguchi)	1	3	Zhang, *et al*.[49]
IMPG2‡	c.1680T>A	p.(Tyr560*)	arRP	1	2	Bandah-Rozenfeld, *et al*.[26]
LCA5‡	c.643del	p.(Leu215Tyrfs*11)	arLCA	1	4	Ahmad, *et al*.[50]
LCA5‡	c.1151del	p.(Pro384Glnfs*17)	arLCA	3	13	den Hollander, *et al*.,[51] McKibbin, *et al*.[38]
MERTK	c.718G>T	p.(Glu240*)	arRP	1	4	Shahzadi, *et al*.[52]
NMNAT1‡	c. 25G>A	p.(Val9Met)	arLCA	1	5	Falk, *et al*.[53]
NMNAT1‡	c.838T>C	p. (*280Glnext*16)	arLCA	1	8	Koenekoop, *et al*.[54]
PDE6A	c.889C>T	p.(Arg256*)	arRP	1	4	Riazuddin, *et al*.[55]
PDE6A	c.1264-2A>G	p.(?)	arRP	1	5	Riazuddin, *et al*.[55]
PDE6A	c.1630C>T	p.(Arg544Trp)	arRP	1	3	Khan, *et al*.[28]

PDE6A	c.2218-2219insT	p.(Ala740Valfs*2)	arRP	1	3	Riazuddin, *et al*.[55]
PDE6B	c.1160C>T	p.(Pro387Leu)	arRP	1	6	Ali, *et al*.[56]
PDE6B	c.1655G>A	p.(Arg552Gln)	arRP	1	9	Ali, *et al*.[56]
PDE6B	c.1722+1G>A	p.(?)	arRP	1	4	Azam, *et al*.[25]
PROM1	c.1726C>T	p.(Gln576*)	arRP	1	6	Zhang, *et al*.[57]
RDH12	c.506G>A	p.(Arg169Gln)	arLCA/arEORD	2	2	Mackay, *et al*.[58]
RDH12	c.619A>G	p.(Asn207Asp)	arLCA/arEORD	1	1	Mackay, *et al*.[58]
RDH5	c.758T>G	p.(Met253Arg)	arCSNB (FA)	1	6	Ajmal, *et al*.[22]
RDH5	c.913_917del	p.(Val305Hisfs*29)	arCSNB (FA)	1	2	Ajmal, *et al*.[22]
RHO	c.448G>A	p.(Glu150Lys)	arRP	2	6	Azam, *et al*.[23]
RHO	c.1045T>G	p.(*349Gluext*52)	adRP	1	8	Bessant, *et al*.[32]
RLBP1	c.346G>C	p.(Gly116Arg)	arCSNB (FA)	1	4	Naz, *et al*.[59]
RLBP1	c.466C>T	p.(Arg156*)	arCSNB (FA)	1	6	Naz, *et al*.[59]
RP1†	c.1118C>T	p.(Thr373Ile)	arRP	2	11	Khaliq, *et al*.[60]
RP1	c.1458_1461dup	p.(Glu488*)	arRP	2	9	Khaliq, *et al*.,[60] Riazuddin, *et al*.[61]
RP1	c.4555del	p.(Arg1519Glufs*2)	arRP	1	5	Riazuddin, *et al*.[61]
RP1	c.5252del	p.(Asn1751Ilefs*4)	arRP	1	4	Riazuddin, *et al*.[61]
RPE65	**c.131G>A**	**p.(Arg44Gln)**	**arEORP**	**1**	**3**	Coppieters, *et al*.,[62] Simovich, *et al*.,[63] **this study**
RPE65	**c.361del**	**p.(Ser121Leufs*6)**	**arEORP**	**1**	**4**	Coppieters, *et al*.,[62] **this study**
RPE65	c.751G>T	p.(Val251Phe)	arLCA	1	6	McKibbin, *et al*.[38]
RPGR	**c.2426_2427del**	**p.(Glu809Glyfs*25)**	**xlRP**	**1**	**8**	Vervoort, *et al*.,[64] (het); **this study**

RPGRIP1	c.587+1G>C	p.(?)	arLCA	1	1	McKibbin, *et al*.[38]
RPGRIP1	c.1180C>T	p.(Gln394*)	arLCA	1	1	McKibbin, *et al*.[38]
RPGRIP1	c.1639G>T	p.(Ala547Ser)	arCRD	3	12	Hameed, *et al*.[65]
RPGRIP1	c.2480G>T	p.(Arg827Leu)	arCRD, arLCA	2	8	Hameed, *et al*.,[65] McKibbin, *et al*.[38]
RPGRIP1	c.3620T>G	p.(Leu1207*)	arLCA	1	1	McKibbin, *et al*.[38]
SAG	c.916G>T	p.(Glu306*)	arCSNB (Oguchi)	1	1	Waheed, *et al*.[66]
SEMA4A	c.1033G>C	p.(Asp345His)	arRP/arCRD	4	4	Abid, *et al*.[67]
SEMA4A	c.1049T>G	p.(Phe350Cys)	arRP/arCRD	4	4	Abid, *et al*.[67]
SEMA4A	c.2138G>A	p.(Arg713Gln) (het)	arRP/arCRD	1	4	Abid, *et al*.[67]
SLC24A1‡	c.1613_1614del	p.(Phe538Cysfs*23)	arCSNB	1	5	Riazuddin, *et al*.[68]
SPATA7	c.253C>T	p.(Arg85*)	arLCA/arRD	2	3	Mackay, *et al*.[69]
SPATA7	c.960dup	p.(Pro321Thrfs*6)	arLCA/arRD	1	6	Mackay, *et al*.,[69] Wang, *et al*.[70]
TTC8†	c.115-2A>G	p.(?)	arRP	1	4	Riazuddin, *et al*.[71]
TULP1	c.1138A>G	p.(Thr380Ala)	arEORP	3	30	Ajmal, *et al*.,[21] Iqbal, *et al*.[72] McKibbin, *et al*.[38]
TULP1	c.1445G>A	p.(Arg482Gln)	arRP	1	8	Ajmal, *et al*.[21]
TULP1	**c.1466A>G**	**p.(Lys489Arg)**	**arRP**	**4**	**19**	Gu, *et al*.,[73] Iqbal, *et al*.,[72] **this study**
ZNF513	c.1015T>C	p.(Cys339Arg)	arRP	1	4	Li, *et al*.,[74] Naz, *et al*.[75]

ACHM, achromatopsia; ad, autosomal dominant; ar, autosomal recessive; CSNB, congenital stationary night blindness; CRD, cone rod dystrophy; EORD, early onset retinal dystrophy; EORP, early onset RP; FA, fundus albipunctatus; het, heterozygous; LCA, Leber congenital amaurosis; RD, retinal dystrophy; RP, retinitis pigmentosa; xlRP, X-linked RP; in bold lettering mutations identified in this study; ‡novel gene identification; †novel phenotype association.

Table 6.4 Mutations identified in Pakistani patients with syndromic retinal dystrophies

Gene	Nucleotide variant	Protein variant	Phenotype	# of families	# of patients	References
AHI1	c.2370dup	p.(Lys791*)	arJBTS	1	2	Utsch, *et al*.[76]
ARL13B	c.236G>A	p.(Arg79Gln)	arJBTS	1	3	Cantagrel, *et al*.[77]
BBS1	c.47+1G>T	p.(?)	arBBS	1	2	Ajmal *et al*.[78]
BBS1	c.442G>A	p.(Asp148Asn)	arBBS	1	2	Ajmal *et al*.[78]
BBS10	c.271dup	p.(Cys91Leufs*5)	arBBS	2	4	White, *et al*.[79]
BBS10	c.1091del	p.(Asn364Thrfs*5)	arBBS	1	1	White, *et al*.[79]
BBS10	c.1958_1967del	p.Ser653Ilefs*4	arBBS	1	2	(Agha *et al*.[80]
BBS10	c.2121dup	p.(Lys708*)	arBBS	1	1	White, *et al*.[79]
BBS12	c.1589T>C	p.(Leu530Pro)	arBBS	2	2	Harville, *et al*.[81]
BBS12	c.2102C>A	p.(Ser701*)	arBBS	1	3	Pawlik, *et al*.[82]
BBS2	c.1237C>T	p.(Arg413*)	arBBS	1	1	Harville, *et al*.[81]
ARL6	c.123+1119del	p.(?)	arBBS	1	1	Chen, *et al*.[83]
BBS5	c.2T>A	p.(Met1Lys)	arBBS	2	2	Harville, *et al*.[81]
TTC8	c.1049+2_1049+4del	p.(?)	arBBS	1	3	Ansley, *et al*.[84]
CC2D2A‡	c.2003+1G>C	p.(?)	arJBTS	1	5	Noor, *et al*.[85]
CDH23‡	c.1114C>T	p.(Gln372*)	arUSH1	1	3	Schultz, *et al*.[86]
CDH23	c.2587+1G>A	p.(?)	arUSH1	1	4	Bork, *et al*.[87]
CDH23	Not mentioned in paper	p.(Arg1305*)	arUSH1	1	4	Bork, *et al*.[87]
CDH23‡	c.3106_3106+11delinsTGGT	p.(Gly1036delinsTrpCys)	arUSH1	1	5	Schultz, *et al*.[86]
CDH23‡	c.6050-9G>A	p.(?)	arUSH1	4	13	Schultz, *et al*.[86]
CDH23‡	c.6050-1G>C	p.(?)	arUSH1	1	6	Schultz, *et al*.[86]
CDH23‡	c.6054_6074del	p.(Val2019_Val2025del)	arUSH1	1	3	Schultz, *et al*.[86]

CDH23‡	c.6845del	p.(Asn2282Thrfs*91)	arUSH1	1	3	Schultz, *et al*.[86]
CDH23‡	c.7198C>T	p.(Pro2400Ser)	arUSH1	1	4	Schultz, *et al*.[86]
CDH23‡	c.8150A>G	p.(Asp2717Gly)	arUSH1	1	3	Schultz, *et al*.[86]
CDH23‡	c.8208_8209del	p.(Val2737Alafs*2)	arUSH1	2	11	Schultz, *et al*.[86]
CEP290	c.5668G>T	p.(Gly1890*)	arJBTS	1	1	Otto, *et al*.,[88] Sayer, *et al*.[89]
IQCB1	**c.488-1G>A**	**p.(?)**	**arSLSN**			**Otto, *et al*.,[31] this study**
IQCB1	c.1465C>T	p.(Arg489*)	arSLSN	1	1	Otto, *et al*.[31]
IQCB1	c.1796T>G	p.(Leu599*)	arSLSN	1	1	Otto, *et al*.[88]
NPHP4	c.3272dup	p.(Ser1092Valfs*11)	arSLSN	1	1	Otto, *et al*.[31]
PCDH15‡	c.7C>T	p.(Arg3*)	arUSH1	1	5	Ahmed, *et al*.[90]
PCDH15‡	c.1927C>T	p.(Arg643*)	arUSH1	1	3	Ahmed, *et al*.[91]
PCDH15‡	c.3389-2A>G	p.(?)	arUSH1	1	3	Ahmed, *et al*.[91]
TCTN2	c.1873C>T	p.(Gln625*)	arJBTS	1	4	Sang, *et al*.[92]
TMEM67	c.647del	p.(Val217Leufs*5)	arMKS	1	2	Smith, *et al*.[93]
TMEM67	c.715-2A>G	p.(?)	arMKS	1	1	Smith, *et al*.[93]
TMEM67	c.1127A>C	p.(Gln376Pro)	arMKS	2	2	Smith, *et al*.[93]
TMEM67	c.1575+1G>A	p.(?)	arMKS	3	5	Smith, *et al*.[93]
USH1G	c.163_164+13del	p.(Gly56*)	arUSH1	1	4	Bashir, *et al*.[94]

ar, autosomal recessive; BBS, Bardet-Biedl syndrome; JBTS, Joubert syndrome; MKS, Meckel syndrome; SLSN, Senior-Loken syndrome; USH1, Usher syndrome type 1; in bold, mutations identified in this study; ‡novel gene identification; †novel phenotype association.

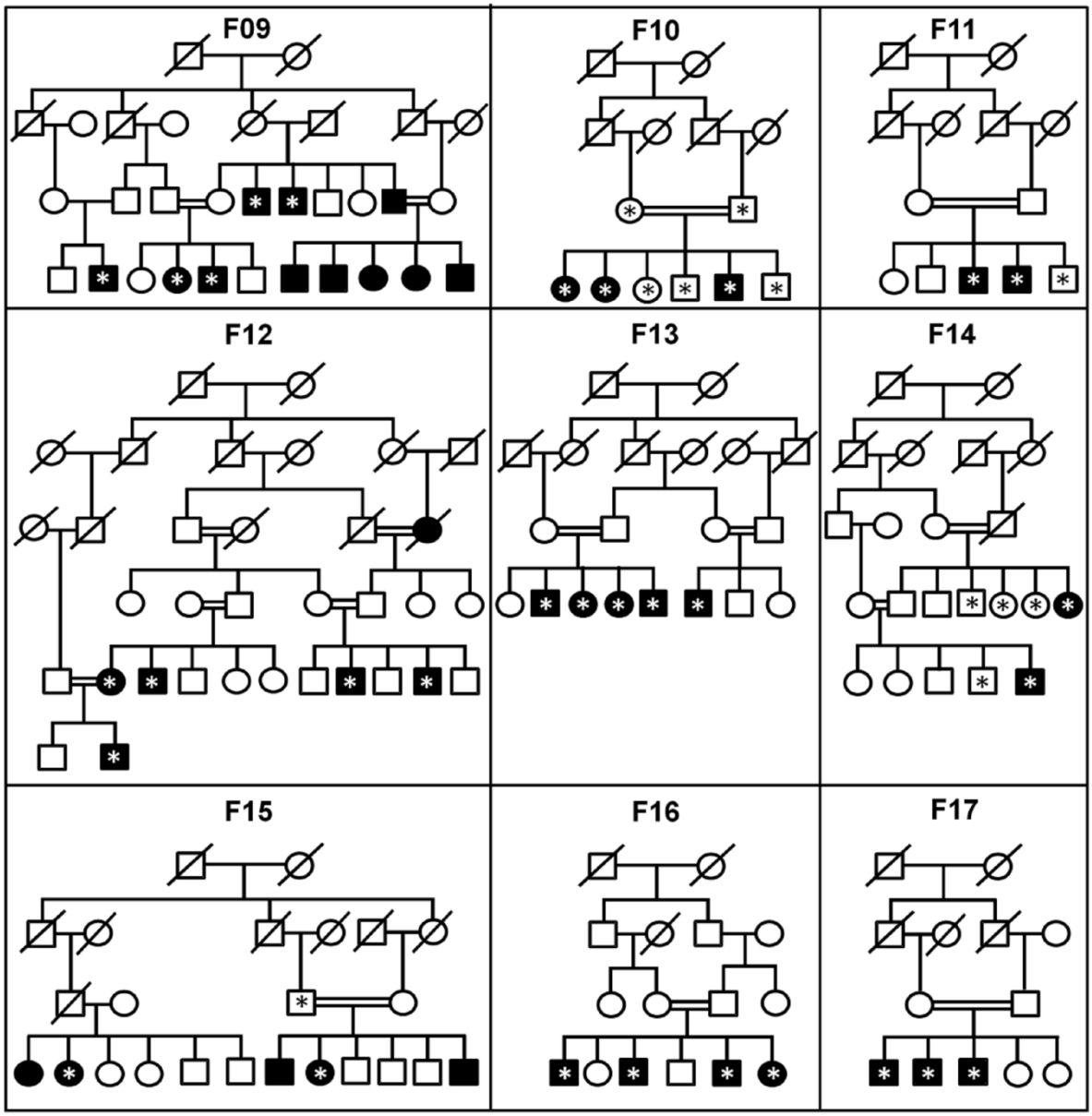

Figure 6.2 Pedigrees of the unsolved arRP families. Black symbols represent affected individuals; open symbols non-affected persons.

Table 6.5 Homozygosity mapping data of families without mutations in genes associated with arRP and LCA

Family	Affected persons	Chr.	Flanking SNPs	Chromosomal positions (in hg19)	Size of Hz regions (Mb)	Ranking Hz regions	LOD Score	Excluded arRP/LCA genes
F09	7	11	rs1825015;rs1944086	36,960,292-63,036,867	26.1	1	4.02	-
		10	rs1986519;rs2181019	101,757,025-108,311,696	17.2	2	3.9	-
		6	rs2050137;rs1416207	139,491,756-143,889,275	4.6	3	2.81	-
		6	rs1367669;rs717796	123,647,417-127,789,979	4.1	4	1.65	-
		12	rs951062;rs3847896	19,988,377-23,159,966	4.1	5	2.04	-
F10	3	1	rs559809;rs6537756	67,035,336-113,099,290	46.1	1	2.7	*ABCA4, RPE65*
F11	2	2	rs1356056;rs744397	170,232,853-178,859,118	8.6	1	1.18	-
		15	rs782944;rs403844	61,338,477-67,296,638	5.9	2	1.90	-
		5	rs923898;rs4702266	2,903,307-5,397,628	2.5	5	1.85	-
F12	5	4	rs894486;rs717614	4,308,513-10,104,788	9.7	1	3.07	*PDE6B*
		16	rs957078; rs163235	14,019,704-20,651,378	6.6	2	1.68	-
		10	rs3852447;rs951308	71,737,065-76,617,479	4.9	3	2.24	-
		12	rs1525895;rs2320519	33,966,496-38,259,123	4.3	4	2.54	-
		6	rs1367669;rs717796	123,647,417-127,789,979	4.1	5	2.31	-
		16	rs724466;rs2188353	22,797,852-26,444,828	3.6	6	1.20	-
		12	rs934159;rs1042503	100,296,887-103,246,700	2.9	8	2.84	-
		14	rs721379;rs1861891	75,902,562-78,509,980	2.6	9	1.30	-
		4	rs1108429;rs1377290	87,165,644-89,719,368	2.5	10	2.30	-
		5	rs723698;rs368337	146,756,993-148,815,759	2.1	11	3.34	-
F13	5	9	rs2254168;rs1045287	113,189,213-130,774,369	17.6	1	2.54	-
		1	rs1768673;rs2136875	220,205,020-226,606,536	6.40	2	2.20	-
		11	rs2156449;rs2001625	126,533,749-130,598,213	4.1	3	2.51	-
		17	rs1062935;rs599314	78,939,857-80,728,072	1.8	4	1.63	*PDE6G*

F14	2	2	rs1015117;rs1567804	86,740,863-101,343,638	14.6	1	1.8	-
		10	rs2227551;rs2039305	75,669,190-89,987,183	14.3	2	1.5	*RGR*
		10	rs7094359;rs6580	92,304,910-105,206,874	12.9	3	1.5	-
		12	rs9300302;rs4883148	3,867,471-8,614,238	4.7	4	1.4	-
F15	5	17	rs1848550;rs1984661	6,745,641-12,650,777	5.9	1	1.8	*AIPL1, GUCY2D*
		21	rs1014803;rs2836301	37,094,040-39,677,589	2.6	3	1.8	-
F16	4	17	rs7946;rs1394385	17,409,560-31,689,618	14.3	1	3.0	-
		4	rs719786;rs1396539	109,396,732-121,108,516	11.7	2	3.0	-
		4	rs1118570;rs963065	96,602,391-102,065,631	5.5	3	3.0	-
F17	3	17	rs11079195;rs8079450	53,904,453-70,355,300	16.4	1	2.4	-
		15	rs11853534;rs2238292	37,033,338-50,551,632	13.5	2	2.4	-
		1	rs4255388;rs10920369	188,770,589-202,203,337	13.4	3	2.4	*CRB1*
		11	rs11222749;rs2212450	99,968,814-112,826,867	12.8	4	2.4	-
		16	rs11646672;rs4783573	66,487,206-68,840,588	2.3	5	2.4	-

Homozygous regions with LOD scores >1.0 are indicated. ar, autosomal recessive; LCA, Leber congenital amaurosis; Mb, mega bases; RP, retinitis pigmentosa; Hz, homozygous. Note: Regions with low LOD scores carry haplotypes that are relatively frequent in the Pakistani population (data not shown).

6.3.3. Overview of molecular genetic studies in non-syndromic RD in Pakistan

Forty-four studies have thus far reported the genetic causes of non-syndromic RD including arCRD, arCSNB, arLCA, and arRP in Pakistani persons, most of which belong to consanguineous families. Together with the results presented here, genetic data of a total of 460 Pakistani RD patients from 106 families (Table 6.3), have been described. Among these retinal phenotypes, arRP was found to be the most frequently occurring RD (64%), followed by arLCA (18%), arCSNB (8%), and arCRD (7%; Table 6.3; Figure 6.3). Autosomal recessive inheritance predominates in the RD families (97%) and only two studies have described mutations in *RHO* (MIM# 180380), and *BEST1* (MIM# 607854), causative of autosomal dominant RP.[32,33] The compiled data showed that out of 119 genes known to be involved in non-syndromic RD, 35 different genes have been reported to be mutated in families of Pakistani origin (Table 6.3; Figure 6.4), reflecting the genetic heterogeneity of the disease in this population. The most frequently mutated genes were *AIPL1* (MIM# 604392), *CRB1*, *SEMA4A* (MIM# 607292), *TULP1*, *RPGRIP1* (MIM# 605446), and *RP1* (MIM# 180100: Figure 6.4). Most of the reported mutations and those we identified in our cohort (Tables 6.2, 6.3 and 6.4), were novel to this population except for mutations in *ABCA4* (MIM# 601691), *CRB1*, *CERKL* (MIM# 608381), *RPE65*, *RPGR* (MIM# 312610), and *SPATA7* (MIM# 609868), which were initially identified in persons of different ethnicity (Table 6.3).

6.3.4. Overview of molecular genetic studies in syndromic RDs in Pakistan

Data of 50 syndromic RD families with a total of 127 affected individuals were collected from 20 studies. Usher syndrome represented about 38% (19/50), of the families in this group, whereas BBS (30%), and MKS (14%), JBTS (10%), and SLSN (8%), accounted for the other families (Table 6.4; Figure 6.5). The most commonly mutated gene associated with syndromic RD in the Pakistani population is cadherin 23 (*CDH23*: MIM# 605516), mutated in persons with Usher type 1, followed by *TMEM67* (MIM# 609884), the gene mutated in persons with autosomal recessive MKS (Table 6.4; Figure 6.6).

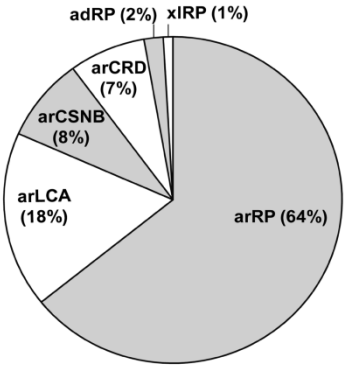

Figure 6.3 Distribution of non-syndromic Pakistani RD families according to their phenotypes. Ad, autosomal dominant; ar, autosomal recessive; CRD, cone-rod dystrophy; CSNB, congenital stationary night blindness; LCA, Leber congenital amaurosis; RP, retinitis pigmentosa; xl, X-linked.

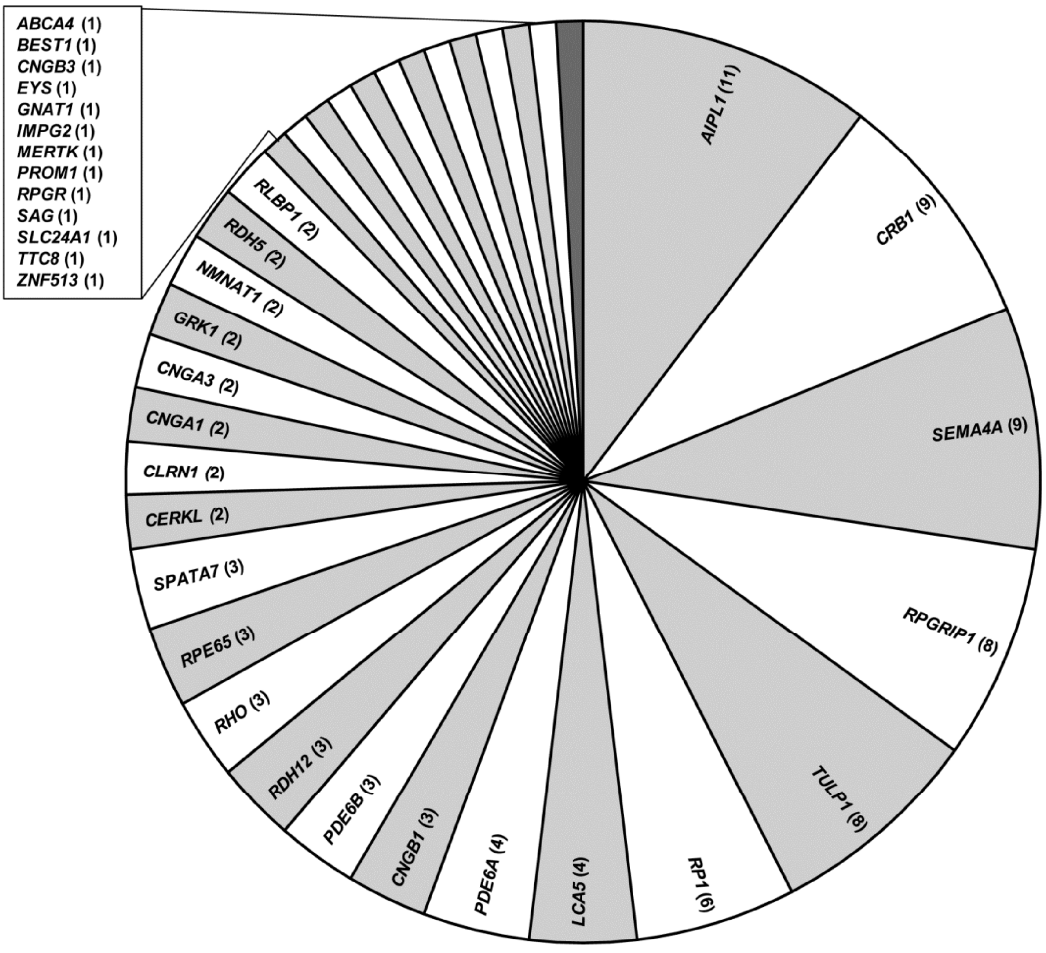

Figure 6.4 Occurrence of gene defects in non-syndromic RD families in Pakistan. Numbers of families with mutations in respective genes are indicated between parentheses.

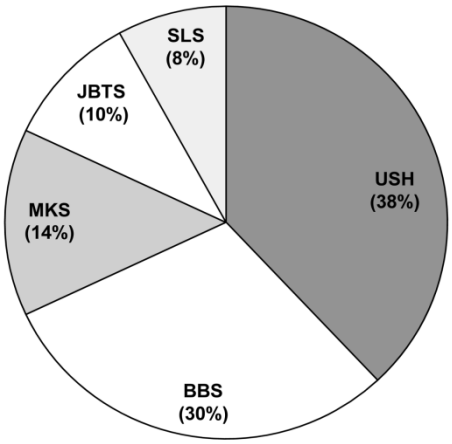

Figure 6.5 Prevalence of syndromic RD phenotypes. BBS, Bardet-Biedl syndrome; JBTS, Joubert syndrome; MKS, Meckel syndrome; SLS, Senior-Loken syndrome; USH, Usher Syndrome.

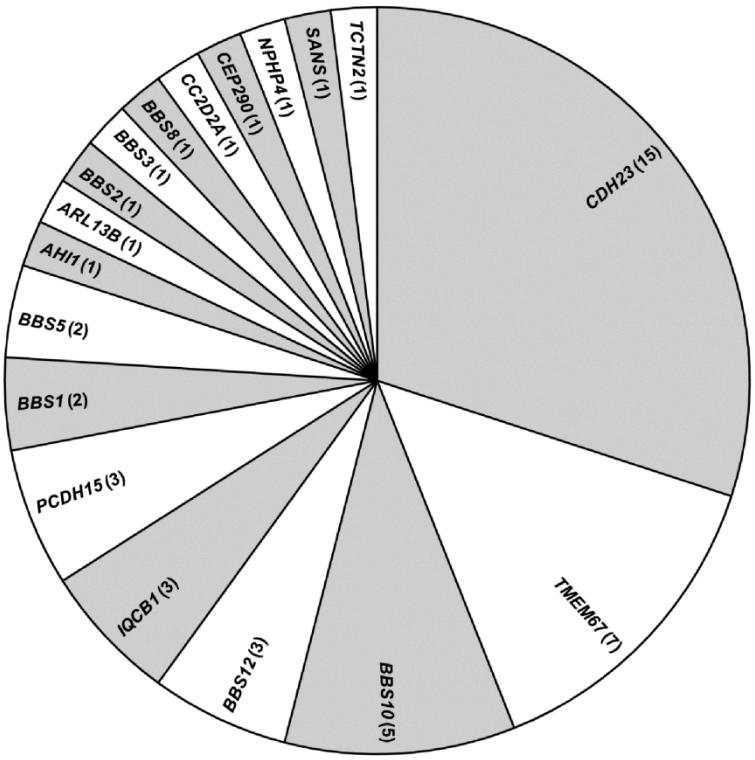

Figure 6.6 Occurrence of gene defects in syndromic RD families in Pakistan. Numbers of families with mutations in respective genes are indicated between parentheses.

6.4. Discussion

Although the Pakistani population is known for its high rate of consanguinity (>60%), but it still is remarkable that 97% of randomly sampled families with inherited RDs had an autosomal recessive inheritance model. It therefore is not surprising that Pakistani families have been instrumental in pinpointing the underlying gene defects through homozygosity mapping.[34,35] Genetic studies of Pakistani families with RD have previously resulted or aided in the identification of ten novel RD genes, i.e. *AIPL1*,[39] *BEST1*,[33] *CC2D2A* (MIM# 612013),[85] *CDH23* (MIM# 605516),[86] *IMPG2* (MIM# 607056),[26] *LCA5* (MIM# 611408),[51] *NMNAT1* (MIM# 608700),[53,54] *ZNF513* (MIM# 613598),[74] *PCDH15* (MIM# 605514),[90] and *SLC24A1* (MIM# 603617).[68] In *CLRN1* (MIM# 606397), and *TTC8* (MIM# 608132), genes previously implicated in the syndromic retinal phenotypes USH3 (MIM# 276902), and BBS (MIM# 209900), causal mutations were identified in persons with non-syndromic arRP.[27,71] In addition, *RP1*, already known to be mutated in adRP, harbored recessive mutations in 3 Pakistani families.[60] Five RD loci including three non-syndromic, i.e. CORD8 (MIM# 605549),[95] RP29 (MIM# 612165),[96] and RP32,[97] and two syndromic, i.e. USH1H (MIM# 612632),[98] and USH1K,[99] have also been identified in Pakistani families.

In the 106 families with non-syndromic RDs that have been reported so far from Pakistan, mutations were most frequently found in *AIPL1* (10%), *CRB1* (8%), *SEMA4A* (8%), *TULP1* (8%), *RPGRIP1* (8%), and *RP1* (6%; Table 6.3; Figure 6.4).

A direct comparison with other RD populations is difficult as comprehensive studies of this kind are rare. A recent study on the screening of Saudi Arabian RD families showed similar results as *RP1* (15%), *TULP1* (12%), *RPGRIP1* (12%), and *CRB1* (9%), were found to be frequently mutated.[100] This should not come as a surprise as these populations share a similar genetic background.[101]

A general literature study revealed arRP-associated mutations in *USH2A* (12%; MIM# 276901), *ABCA4* (8%), *PDE6B* (7%; MIM# 180072), *CNGB1* (6%), and *PDE6A* (5%; MIM# 180071).[102] In a more recent study of 230 Dutch persons with isolated or arRP,[103] the most frequently mutated genes were *EYS* (11%; MIM# 602772), and *CRB1* (11%), followed by *USH2A* (10%), *ABCA4* (9%), and *PDE6B* (7%). The absence of *USH2A* variants in Pakistani patients is probably due to the fact that the most frequent arRP-associated variant, (c.2299delG; p.(Glu767Serfs*21)), invariably is found in compound heterozygous states with the second mutation that is considered to be mild,[104] precluding its detection in a

homozygosity mapping approach. Other differences can only be attributed to divergent genetic backgrounds of these populations.[105,106]

The four most frequent variants, p.(Trp278*) in *AIPL1*, p.(Lys489Arg) in *TULP1*, p.(Asp345His), and p.(Phe350Cys) in *SEMA4A* (Table 6.3), explain about 22% of the non-syndromic Pakistani RD families. The p.(Trp278*) variant has been identified as the most frequent *AIPL1* variant in many LCA studies,[107,108] suggesting that this variant is relatively old. Although 108/115 variants listed in Tables 6.3 and 6.4 have only been identified in Pakistani patients, 8 variants (*LCA5*, p.(Pro384Glnfs*17); *RPGRIP1*, p.(Ala547Ser) and p.(Arg827Leu); *SEMA4A*, p.(Asp345His) and p.(Phe350Cys); *TULP1*, p.(Thr380Ala); *TMEM67*, c.1575+1G>A and p.(Gln37Pro)), are more frequent than others and have not been identified in other studies. We therefore consider them to be population-specific. Likewise, variants such as p.(Met390Arg) in *BBS1* (MIM# 209901), and p.(Cys91Leufs*5) in *BBS10* (MIM# 610148), that account for most of the BBS cases in the French population,[109] were not found in Pakistani BBS cases. The 8 frequent variants mentioned above, together with 5 other variants *RDH12* (MIM# 608830), p.(Arg169Gln); *RHO*, p.(Glu150Lys); *RP1*, p.(Thr373Ile), p.(Glu488*), *SPATA7*, p.(Arg85*), account for approximately 42% of all non-syndromic RD families in Pakistan. A cost-effective initial genetic screening for Pakistani persons with RD therefore could be to analyze these variants or a larger subset of variants such as listed in Tables 6.3 and 6.4, using arrayed primer extension (APEX) analysis or other allele-specific genotyping methods[110-112] that could be specifically designed for the Pakistani population.

By employing the approach of homozygosity-mapping and subsequent sequence analysis of known RD-associated genes residing in homozygous regions, 32 out of 41 (78%), families have been solved (Figure 6.7). These include 17 families (8 solved, 9 unsolved), which are described in here and 24 families solved previously.[19-28]

In a total of 26 out of 32 families we performed homozygosity mapping and subsequent sequence analysis of candidate genes previously implicated in inherited retinal diseases. In most of these families 96% (i.e. 25/26), the linked region contained the known retinal disease gene that harbored the pathogenic mutations (Tables 6.2 and 6.3). The causative mutation in 22 of 26 families (85%) was found in the largest homozygous region, which is not unexpected as the largest regions generally contain the largest number of genes. Sequence analysis of previously identified RD-associated genes that are located in homozygous regions was successful in 31 of 40 (78%), families with presumed autosomal recessive inheritance.

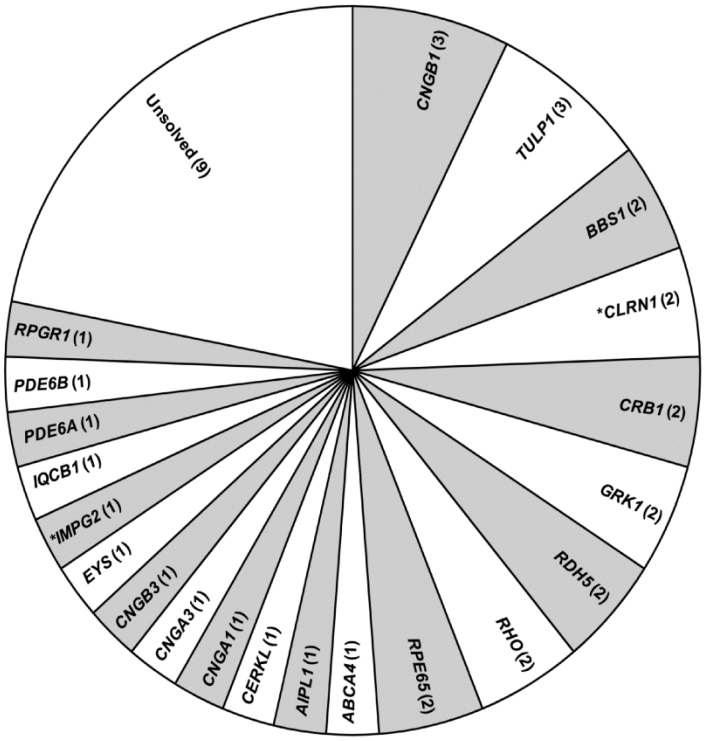

Figure 6.7 Genes mutated in current cohort of 41 families. Numbers of families with mutations in respective genes are indicated between parentheses. *Novel autosomal recessive RP genes identified in this cohort.

In a comparable study of multiplex families, 36/63 (57%) were solved using a similar approach.[100]

Employing homozygosity mapping and candidate gene analysis, the genetic cause could not be determined in 9 families with arRP (Figure 6.2, Table 6.5). For these families LOD scores of homozygous regions varied from 1.8 to 4.0. Highly suggestive linkage (LOD >2.7), was found for two families (F10 and F16), and significant LOD scores (>3.3), were found for F09 and F12. However, only in family F10 a single homozygous region of 46.1 Mb was found on chromosome 1 (LOD score 2.7), which was found to be overlapping with the RP32 locus (MIM# 609913).[97] The remaining five families showed multiple homozygous regions due to a smaller number of affected individuals. In each of these families, the homozygous regions can harbor the causative mutation, but we cannot exclude other possibilities. First, we cannot rule out deep-intronic causal variants in the 'excluded' RD-associated genes. Second, we assume that affected individuals from these consanguineous families carry homozygous mutations but, considering that there is consanguinity in ~60% of Pakistani families,[11] compound heterozygous mutations may also be present in consanguineous families. Similarly, X-linked inheritance also can be suspected in families with a preponderance of affected males and no

male-to-male transmission of gene defects. Indeed, among 6 tested families (F03, F06, F08, F11, F12 and F17), with these characteristics, we identified a causal *RPGR* mutation in one family (F03; Figure 6.1).

Our systematic approach yielded causal mutations in 78% (32/41), of families with inherited syndromic and non-syndromic RDs, suggesting for the first time that the majority of causal genes for inherited RDs have been identified. The next steps in our analyses will be the exclusion of mutations in genes previously implicated in allied syndromic and non-syndromic retinal diseases, and if not successful, next generation sequencing of the respective exomes.

In conclusion, we have provided a comprehensive overview of genetic causes of non-syndromic and syndromic retinal diseases in Pakistan, the results of which can be used to design a cost-effective screening platform for future genetic testing in Pakistan. In consanguineous families homozygosity-directed sequence analysis facilitates mutation identification. In addition, under the assumption that homozygous mutations underlie the retinal phenotypes, we have identified novel candidate arRP loci in 9 families, which will facilitate the identification of novel causal genes.

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Chapter 7

Exome sequencing identifies a *DHX38* missense mutation associated with severe early-onset retinitis pigmentosa in a Pakistani family: a new RNA splicing gene defect associated with a macular coloboma.

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ABSTRACT

Background: Retinitis pigmentosa (RP) is the most frequent inherited retinal disease, which shows a relatively high incidence of the autosomal recessive form in Pakistan. By combining several genotyping methods, we investigated the underlying genetic defects in a consanguineous Pakistani family with RP.

Methods: Genome-wide high density single nucleotide polymorphism (SNP) microarrays were used to identify homozygous regions shared by affected individuals of the family. DNA of three affected and two healthy siblings was used for SNP genotyping. Genotyping data were then analyzed by Homozygosity Mapper, an online data analysis tool. DNA of the Proband (IV-1) was further analyzed employing exome sequencing.

Results: Data analysis with Homozygosity Mapper revealed a single significant homozygous region on chromosome 16 shared by the 4 affected individuals that did not harbor a gene previously implicated in any retinal dystrophy. Linkage analysis yielded a LOD score of 2.65, which is highly suggestive for linkage. Subsequent exome sequencing identified a novel missense mutation, c.995G>A; p.(Gly332Asp), in *DHX38*. This mutation was found to be present in a homozygous state in four affected individuals while healthy siblings and parents of the affected persons were heterozygous for this mutation. This variant was neither detected in 180 ethnically matched control individuals, nor in an in-house database that contained the exome data of 400 individuals.

Conclusions: By combining genome-wide homozygosity mapping and exome sequencing, a novel missense mutation was identified in the *DHX38* gene that encodes the pre-mRNA splicing factor PRP16, in a Pakistani family with early-onset autosomal recessive RP. The phenotype is different from those associated with other retinal mRNA splicing factors. This is the first time a mRNA splicing gene is associated with recessive RP.

Key words: Homozygosity mapping; exome sequencing; retinitis pigmentosa; *DHX38*; consanguineous; PRP16.

7.1. Introduction

Retinitis pigmentosa (RP) (MIM268000) is a genetically and clinically heterogeneous disease, and one of the most frequent subtypes of retinal dystrophy.[1,2] Patients with classical RP usually experience night blindness in their teens, followed by daytime vision defects (tunnel vision), and later central vision loss which, in some cases, may result in complete blindness. Rod photoreceptors are affected first, but gradually cone photoreceptors also die. Funduscopic characteristics are retinal bone spicules with atrophic areas, attenuation of retinal vessels, and optic disc pallor. In patients with early-onset RP (also called juvenile RP or early onset retinal dystrophy or EORD), symptoms appear in the first six years of life, and often it is not easy to distinguish early-onset RP from Leber congenital amaurosis (LCA), which comprises congenital onset retinal dystrophy.[2]

RP displays an unusually high degree of allelic and genetic heterogeneity for a Mendelian disorder as eight loci and 55 different genes have thus far been identified that are known to cause non-syndromic RP (https://sph.uth.tmc.edu/retnet/). Approximately 70% of cases are due to autosomal recessive defects, 20% due to autosomal dominant defects, and 10% due to X-linked defects.[3,4] Our goal is to identify the remaining causal mutated genes for RP, for LCA and the other retinal dystrophies.

The gene encoding DEAH (Asp-Glu-Ala-His) box polypeptide 38 (*DHX38*: accession# NM_014003.3) is located on the long arm of chromosome 16 (q22.2) and consists of 26 protein-coding exons. *DHX38* encodes an ATP-dependent RNA helicase precursor mRNA processing factor 16 (PRP16) that contains 1227 amino acids. Human PRP16 is known to be involved in pre-mRNA splicing.[5]

In this study, we identified the genetic cause of autosomal recessive early-onset RP in a Pakistani family and revealed a novel missense mutation in *DHX38* associated with early-onset RP using homozygosity mapping followed by exome sequencing.

7.2. Methods

7.2.1. Ethics committee approval and patient consent

Approval for this study was granted by the ethics committee/institutional review board of Shifa College of Medicine and Shifa International Hospital, Islamabad, Pakistan. Approved Informed consent was obtained from participating individuals.

7.2.2. Patient selection

A family segregating autosomal recessive RP (Figure 7.1A) was residing in a remote area of the Khyber Pakhtunkhwa province of Pakistan. Four affected and four healthy individuals of the family (Figure 7.1A) were included in the study. The proband was clinically evaluated and fundus photographs were taken (Figure 7.1B). Electrophysiological responses of rod and cone photoreceptors were also recorded according to the International Society for Clinical Electrophysiology of Vision (ISCEV) protocol.[6]

7.2.3. Blood collection and DNA extraction

Blood samples were collected in vacutainers containing acid citrate dextrose. A standard phenol/chloroform extraction method was used to isolate DNA.[7]

7.2.4. Homozygosity mapping analysis

The DNA of two healthy (IV-3 and IV-6) and three affected (IV-1, IV-2 and IV-4) siblings of the family were selected for genotyping with HumanOmniExpress (700K) SNP microarrays, from Illumina Inc. (San Diego, CA). Data obtained from SNP microarrays were analyzed by an online tool, Homozygosity Mapper[8] to identify homozygous regions shared by the affected individuals.

7.2.5. Linkage analysis

The Gene Hunter v2.1r5 program in the easyLINKAGE plus v5.08 software package was used to calculate the logarithm of the odds (LOD) scores for the identified homozygous regions.[9]

7.2.6. Exome sequencing

The proband's (IV-1) DNA was subjected to exome sequencing to identify the underlying genetic cause of the disease. Massively parallel sequencing of genomic DNA from the affected individual was performed on a SOLiD^{TM} 4 sequencing platform (Life Technologies, Carlsbad, CA). Enrichment of exonic sequences was achieved by using the SureSelect^{XT} Human All Exon v.2 Kit (50Mb), containing the exonic sequences of ~21.000 genes (Agilent Technologies, Inc. Santa Clara, CA). Color space reads were mapped to the hg19 reference genome with SOLiD LifeScope software version 2.1 (Life Technologies, Carlsbad, CA), which uses an iterative mapping approach. Single-nucleotide variants were subsequently

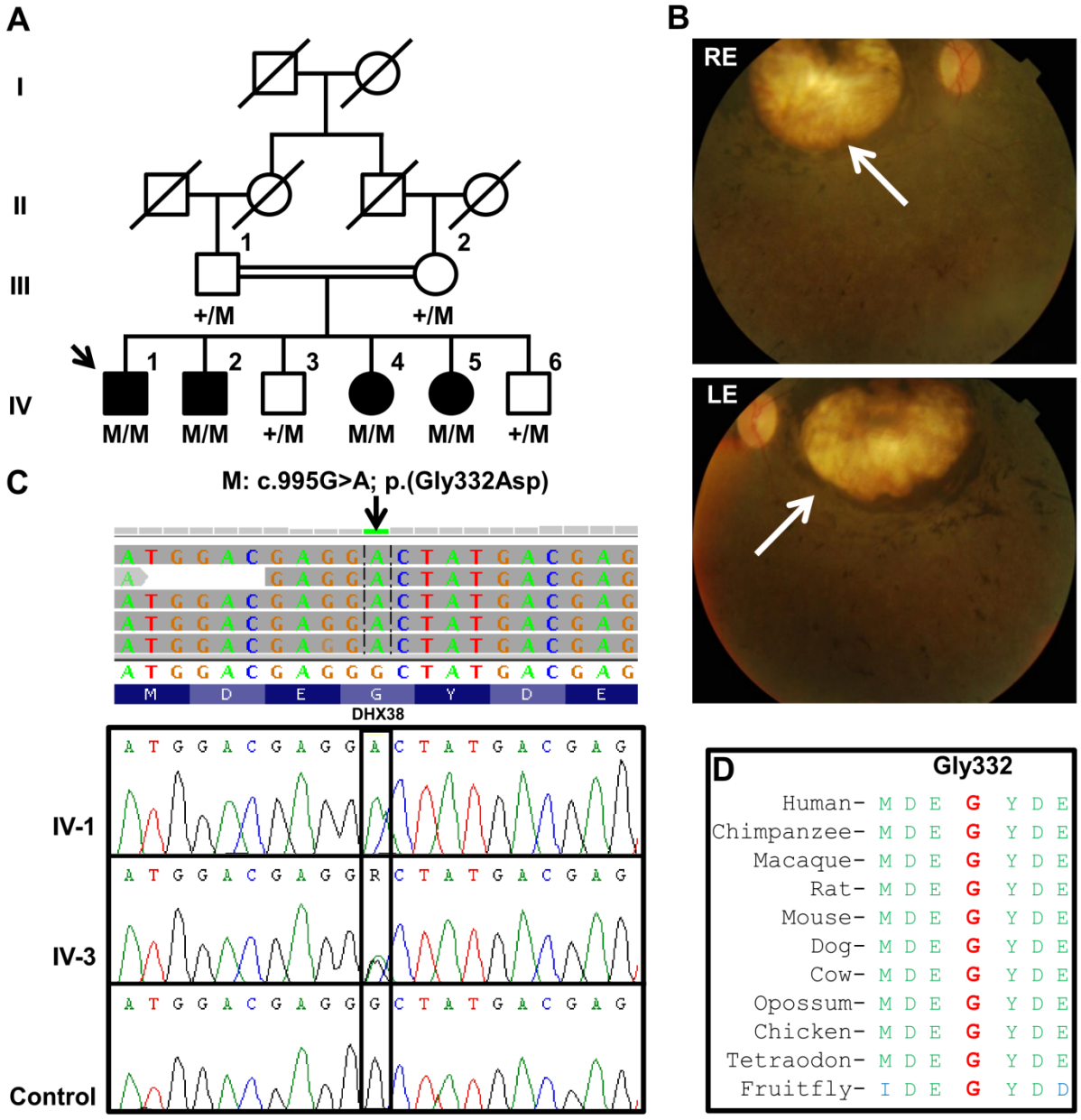

Figure 7.1 Genetic and clinical findings in the family. A. Pedigree of the family along with segregation of the mutant allele. **B.** Fundus photographs (left and right eye) of proband presenting vascular constriction, bone spicules and macular atrophy in both eyes. White arrows indicate macular colobomas. **C.** Mapped reads with mutant adenine revealing the c.995G>A variant (upper panel) and electropherograms of affected individual (IV-1), sibling carrying the heterozygous variant (IV-3), and a healthy control. **D.** Alignment of human DHX38 protein sequence (from amino acid 329 to 335) to its orthologous protein sequences in different species indicating evolutionary conservation of arginine at position 332 in human DHX38.

called by the DiBayes algorithm using high-stringency calling settings, and small insertions and deletions were detected using the SOLiD Small Indel Tool.

Variant prioritization was performed as described previously.[10] In brief, we selected private non-synonymous exonic and canonical splice site variants, *i.e.* we selected for variants that

are not included in dbSNP135 and our in-house database (including 400 exomes at the time of analysis). Furthermore, under the assumption of the presence of homozygous variants, we only selected variants that presented in at least 80% of all reads.

7.2.7. Candidate gene sequencing analysis

PCR primers for *DHX38* and other genes, for which validation by Sanger sequencing was required, were designed by Primer3 online software.[11] PCR reactions were performed using standard conditions. Amplified PCR products were electrophoresed to check the amplification and then purified using PCR purification plates (NucleoFast™ 96 PCR, Cat. No. 743100.10; Macherey Nagel, Düren, Germany), according to the manufacturer's protocol. Sequencing was done with an automated DNA sequencing platform (3730 DNA analyzer; Applied Biosystems, Inc., Foster City, CA).

7.2.8. Prediction tools

The pathogenicity of the missense variant identified in the family was analyzed using the online prediction tools Sorting Intolerant from Tolerant (SIFT) (http://sift.jcvi.org/www/SIFT_enst_submit.html) and Polymorphism Phenotyping v2 (PolyPhen-2) (http://genetics.bwh.harvard.edu/pph2/).

7.2.9. Protein conservation study

DHX38 orthologous protein sequences from different vertebrate and invertebrate species were aligned to study the evolutionary conservation of mutated amino acid p.(Gly332Asp) using Vector NTI Advance™ 2011. *Homo sapiens* (NM_014003), *Pan troglodytes* (ENSPTRP00000014240), *Macaca mulatta* (ENSMMUP00000000912), *Rattus norvegicus* (ENSRNOP00000019810), *Mus musculus* (ENSMUSP00000047865), *Canis familiaris* (ENSCAFP00000030010), *Bos taurus* (ENSBTAP00000042866), *Monodelphis domestica* (ENSMODP00000003098), *Gallus gallus* (ENSGALP00000037931), *Tetraodon nigroviridis* (ENSTNIP00000004088), and *Drosophila melanogaster* (FBpp0073717) were included in the alignment.

7.2.10. Protein structure prediction

For the assessment of possible structural changes in the mutant protein, the HOPE tool was used.[12] Wild-type human protein PRP16 sequence (Accession number Q92620) encoded by

DHX38 was used to predict the mutant protein structure.

7.2.11. Estimation of non-coverage in the known arRP/LCA genes

To estimate the non-coverage in exome sequencing of known genes implicated in arRP/LCA, colour space reads were visually checked in binary alignment/map format (BAM) file.

7.3. Results

7.3.1. Clinical findings

The age of onset of RP in the family was four years in all affected individuals. The ophthalmic examination of the proband's eyes revealed severely attenuated retinal vessels throughout the entire fundus. The maculae of both eyes were severely affected showing unusually prominent and deep macular colobomas devoid of neuro-retinal tissue (Figure 7.1B). All affected individuals were completely blind (no light perception). Electrophysiological responses of rod and cone photoreceptors of the proband were also severely reduced (Table 7.1).

Table 7.1 Comparison of electrophysiological responses of rod and cone photoreceptors of proband and a healthy individual.

Measured parameters using monopolar electrodes	Adaptation	Flash strength (cd.s/m^2)	Proband (IV-1)*	Healthy Brother (IV-6)	Standard Values (Age=30 years)
Scotopic 25 dB b-wave amplitude (μV)	Dark	0.01	33.1	250.6	>185
Scotopic 0 dB b-wave amplitude (μV)	Dark	3.0	11.0	533.4	>419
Oscillatory potential amplitude (μV)	Dark	3.0	18.4	337.8	>110
Photopic 0 dB b-wave amplitude (μV)	Light	3.0	18.3	135.8	>102
Photopic 30 Hz flicker amplitude (μV)	Light	3.0	2.1	87.7	>70

*Age of the proband was 22 years at the time of testing.

7.3.2. Genetic findings

High-density SNP microarray data analysis with Homozygosity Mapper yielded a single homozygous region of 10.9 Mb (hg19: 63.8 Mb - 74.7 Mb) defined by rs10500477 and rs12598704 on chromosome 16 (Figure 7.2). Employing SNP data from three affected and two unaffected individuals, multipoint LOD score of 2.65 was obtained by Gene Hunter

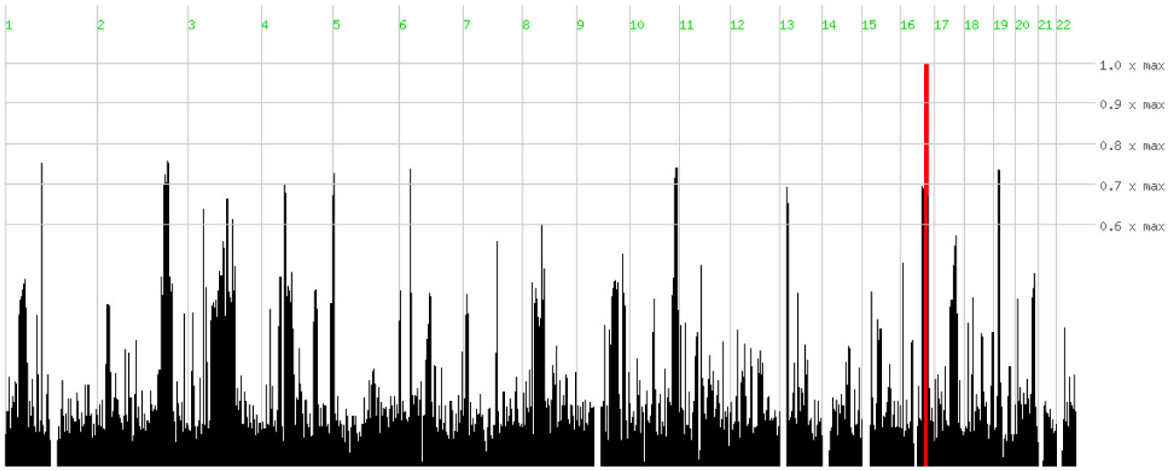

Figure 7.2 Homozygosity mapping results. Single homozygous region identified in the family is indicated by red line in the Homozygosity Mapper plot.

v2.1r5 with marker rs11646282 (72.6 Mb), which is highly suggestive for linkage.

In total, 29,873 variants were called as a result of exome sequencing, details of which are given in Table 7.2. Filtering the data allowed us to narrow down the huge list of variants to only two homozygous variants in two different genes, *i.e.*, *HYDIN* and *DHX38*. The variant in *HYDIN*, c.4460T>C; p.(Glu1487Gly), was not confirmed by Sanger sequencing whereas the variant in *DHX38*, c.995G>A; p.(Gly332Asp) was confirmed. Sequence analysis (Figure 7.1C) of this *DHX38* variant further confirmed the co-segregation with the disease phenotype in the family. All affected siblings were homozygous for the mutant allele; parents and other normal individuals were carriers of one mutant allele (Figure 7.1A). The *DHX38* variant p.(Gly332Asp) was highly conserved in different vertebrate and invertebrate species (Figure 7.1D). The wild-type nucleotide guanine at cDNA position 995 is also highly conserved, with a PhyloP score of 5.34. SIFT predicted p.(Gly332Asp) as “deleterious” whereas Polyphen-2 predicts this variant as “probably damaging” with a score of 0.998.

Analysis of project HOPE[12] predicted that the wild-type residue glycine at position 332 is the most flexible of all residues, and this flexibility might be necessary for the protein's normal function. The mutant aspartic acid at this position can abolish this flexibility and thereby result in an impaired function of the protein. Moreover, the mutation introduces a charge, which can cause repulsion of ligands or other residues with the same charge. The size of the aspartic acid is also bigger than the wild-type glycine residue, which might lead to sterical hindrance. Because of the flexible nature of the glycine residue it is possible that this residue is needed at this position to make a special backbone conformation or to facilitate movement of the protein.

Table 7.2 Detailed summary of exome variants identified in proband's DNA

Type of variant	Quantity
Total variants identified	29,873
Variants in genes	23,680
Exonic variants	14,130
Splice site variants	1,645
Variants in canonical splice sites	34
Variants in micro RNA	0
Variants in SNPs	27,449
Nonsense variants	47
Missense variants	6,152
Synonymous variants	6,817
Transitions	19,653
Transversions	7,585
Deletions	1,490
Insertions	1,144
Homozyous (>0.95)	9,691
Variants on chromosome X	470
Variants on chromosome Y	304
Variations of 1 bp	28,025
Variations of 2 bp	217
Variations of 3 bp	184
Variations of 4 bp and more	7

The identified variant was not present in 180 ethnically matched healthy controls nor is it present in the exome variant server (EVS) database (http://evs.gs.washington.edu/EVS/), the 1000 genomes project (1092 exomes; (www.1000genomes.org/) and dbSNP (www.ncbi.nlm.nih.gov/projects/SNP/).

To exclude an involvement of compound heterozygous changes located outside the homozygous regions, we performed an unbiased analysis of the exome data which resulted in only one pair of compound heterozygous mutations, e.g. in *SRRM2*. These two variants in *SRRM2* (c.1001C>T; p.(Pro334Leu) and c.5551C>T; p.(Arg1851Cys)) were confirmed in the proband by Sanger sequencing, but did not co-segregate with the disease in the family.

Manual visualization of BAM file revealed that 57 (~8%) (Table 7.3) coding exons of known genes implicated in arRP/LCA were not covered in the exome sequencing.

We also screened seven additional probands from different families (Table 7.4) in which significant overlapping homozygous regions encompassing *DHX38* were identified but Sanger sequencing did not identify any causative mutation. Hence the variant identified in our family might be a private variant, which is causing the severe and unusual early onset RP in this family.

Table 7.3: Details of uncovered exons in known genes implicated in arRP/LCA

Gene	Protein coding exons	Uncovered exons
ABCA4	50	4, 30
AIPL1	6	-
BEST1	8	3, 9
C2ORF71	2	1, 2
C8orf37	6	-
CABP4	5	1
CEP290	53	-
CERKL	14	1
CLRN1	4	3
CNGA1	8	1, 2
CNGB1	32	1, 10, 33
CRB1	12	-
CRX	3	1
DHDDS	8	-
EYS	40	15, 23, 26-32, 42
FAM161A	6	-
GUCY2D	18	2, 3, 4, 11, 16-19
IDH3B	12	-
IMPG2	19	1
IQCB1	13	-
KCNJ13	2	-
LCA5	7	-
LRAT	2	-
MAK	13	-
MERTK	19	-
NMNAT1	4	-

NR2E3	9	1, 2, 3, 4
NRL	2	3
PDE6A	22	-
PDE6B	22	1, 16
PDE6G	3	-
PRCD	3	1, 4
PROM1	26	-
RBP3	4	1
RD3	2	3
RDH12	7	-
RGR	7	-
RHO	5	-
RLBP1	7	-
RP1	3	-
RPE65	14	-
RPGRIP1	24	-
SAG	15	-
SNRNP200	45	5, 8-12, 30, 32
SPATA7	12	-
TTC8	15	-
TULP1	15	3, 4, 13
USH2A	71	-
ZNF513	3	2
Total	702	57

Table 7.4 Summary of overlapping regions in seven probands along with ethnicity and phenotype.

Proband	Size of IBD region (in Mb)	Ranking	Phenotype
Dutch	21.1	6	CRD
French	25.0	10	arRP
Israeli	22.0	2	arRP
Canadian	21.2	6	arRP
Canadian	21.9	1	arRP
Belgian	34.0	3	USH2
Belgian	61.5	1	LCA

7.4. DISCUSSION

Here, we report a Pakistani family with early-onset RP that carries a novel missense mutation c.995G>A; p.(Gly332Asp) in *DHX38*, which was identified using homozygosity mapping followed by exome sequencing. *In-silico* prediction tools SIFT and Polyphen results indicated that this mutation is a potentially pathogenic mutation, and might affect the normal topology of the protein as predicted by HOPE analysis. This variant segregated perfectly in the family and was also absent in 180 ethnically matched controls and 1492 exomes of persons with several other inherited diseases. Exome sequencing ruled out mutations in approximately 90% of exonic sequences of known retinal dystrophy genes, both in a homozygous state (in homozygous regions >2Mb) or in a compound heterozygous state. Together, these results strongly suggest that *DHX38* might be a rare and novel cause of severe early-onset RP in Pakistani population.

DHX38 belongs to the DEAD/DEAH box family of proteins and this family consists of 57 members.[13] DEAD/DEAH box family proteins are involved in a diversity of cellular processes including pre-mRNA splicing, spliceosome assembly,[5] modification of RNA secondary structure,[14] cellular growth and ribosomal RNA synthesis.[13] *DHX38* encodes the pre-mRNA splicing factor PRP16 that is involved in catalyzing the second step in pre-mRNA splicing,[15,16] as it removes the lariat and joins two exons. PRP16 binds and interacts with two other splicing factors, G-patch domain and KOW motifs-containing protein (GPKOW), and hPRP2, during the second step of pre-mRNA splicing.[17] Prp16 has also been reported to have an ATP-independent function in the first step of pre-mRNA splicing.[18] In the first catalytic step yeast Prp16 has a stabilizing effect on the Cwc25 binding to the spliceosome to assist in the splicing of branch point mutant pre-mRNAs.[18] Mutations in five other spliceosomal factors, e.g. precursor mRNA processing factor 3 (*PRPF3*),[19] precursor mRNA processing factor 6 (*PRPF6*),[20] precursor mRNA processing factor 8 (*PRPF8*),[21] precursor mRNA processing factor 31 (*PRPF31*)[22] and small nuclear ribonucleoprotein 200kDa (*SNRNP200*),[23] are associated exclusively with autosomal dominant RP. *SNRNP200* encoded Brr2p interacts with Prp16 and assists the binding of Prp16 to the spliceosome.[24] This interaction may be affected by mutations in *DHX38*. Interestingly, we recently identified for the first time an autosomal recessive missense variant in *SNRNP200* in two affected individuals from a consanguineous family with RP (M.I.K. *et al*. unpublished). This variant, p.(Arg1090Gln), affects the same amino acid residue of SNRNP200 that previously was found to be mutated in an autosomal dominant family with RP.

Interestingly, splicing factor genes are widely expressed but mutations in some of these genes exclusively result in retinal dysfunction. A possible explanation is that the vision process due to the constant photoreceptor outer segments renewal (10% of discs per day), requires large amounts of rhodopsin and other retinal proteins and that this requires higher levels of pre-mRNA splicing as compared to other cell types.[17,23] Alternatively, the splice factors thus far implicated in adRP might have a specific role in retinal cells by splicing a subset of pre-mRNAs.[17] Finally, it is also possible that other factors may compensate for the absent or lower activity of PRP16 in non-retinal cells during pre-mRNA splicing. The identification of a recessively inherited *DHX38* mutation in the current study is of interest as it might shed further light on the retinal disease mechanism underlying dysfunctional pre-mRNA splicing.

The mutated amino acid residue aspartic acid has different biochemical properties as compared to wild type glycine that might result in misfolding of the mutant protein. Misfolded proteins are mostly degraded to protect the cell from toxic effects of misfolded proteins.[25-27] Despite all these arguments future genetic studies are required to identify additional autosomal recessive RP families with *DHX38* variants to further strengthen the evidence of pathogenicity. Additional functional studies in cell systems and animal models of the disease might confirm the pathogenic nature of this *DHX38* variation.

Although exome sequencing is becoming popular and growing at a very fast pace it has coverage issues as ~8% exons of the known retinal disease genes were not covered. Hence, in families with new discoveries there is still a chance that variants in the known genes might have been missed because of coverage limitation of the technology. By comparing the SNP haplotypes of all affected individuals, we have excluded the involvement of these uncovered exons in this study, but this will most likely not be done on a routine basis. Another issue is that exome sequencing is detecting variations in the exonic and flanking intronic regions whereas deep intronic variations are missed.

In conclusion, we identified a novel missense mutation in *DHX38* in a Pakistani family with affected siblings employing homozygosity mapping and exome sequencing. Future research on *DHX38* will further elaborate the molecular mechanisms responsible for the severe early onset of RP in this family, and improve our understanding on how dysfunction of spliceosome components result in non-syndromic retinal dystrophy.

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Chapter 8

General discussion

In this thesis, a systematic approach of homozygosity mapping, dedicated Sanger sequencing of genes previously implicated in retinal disease and residing in significant (>2 Mb) homozygous regions, and exome NGS of unresolved families was employed. In this manner, disease-causing mutations were identified in 19 different families of which seven were novel mutations in known genes, eleven were previously described mutations and one potentially pathogenic missense mutation was found in a gene that has not yet been associated with retinal disease.

Below, I will discuss the importance of the clinical evaluation of patients as a major decision-oriented step in identifying the underlying genetic cause in inherited eye diseases. After this, homozygosity mapping and exome sequencing will be discussed as a powerful tool to be used in research as well as molecular diagnostics. In further sections, I will discuss: allelic heterogeneity, phenotype variability, correlations of different genotypes with specific phenotypes, the possibility of therapeutic intervention, linking clinicians and geneticists, and benefits to the patients and their families. In the last section future research directions will be discussed.

8.1. Clinical evaluation, a decision making step towards genetic testing

One of the major hurdles in this study was to obtain reliable clinical data of the affected individuals residing in remote areas of the Punjab and Khyber Pakhtunkhwa provinces of Pakistan. Good clinical data are necessary to facilitate the genetic testing of an inherited disorder. In the case of retinal degenerations, electrophysiological responses of the retinal photoreceptors are required to properly diagnose the subtype of retinal degeneration. There are a limited number of institutes in Pakistan that offer facilities for these diagnostic tests, which are mainly situated in major cities. Most of the families included in this study live in remote areas; therefore, only one affected individual was selected from each family for detailed clinical evaluation

The clinical assessment of patients with eye disorders is mostly based on different diagnostic tests like the measurement of visual acuity (VA), the visual field (VF), fundus examination, and electroretinography (ERG). In addition, the age of the patient at the time of clinical evaluation is also important to distinguish between clinical subtypes of some retinal dystrophies (RD). Progressive and non-progressive subtypes of RD can only be differentiated when the diagnosis is made early and follow-up examinations are well planned and documented.[1] Families described in the **third chapter** are a good example of the importance

of a regular clinical follow-up testing to distinguish between fundus albipunctatus (FA) and retinitis punctata albescens (RPA). FA and RPA are distinguishable by ERG as rod photoreceptors of FA patients exhibit delayed dark adaptation but rods of RPA patients fail to recover. In addition, RPA is a progressive disease whereas FA is considered as stationary and is a subtype of congenital stationary night blindness (CSNB).[2] Similarly, in the case of syndromic retinal diseases, additional diagnostic tests are needed to establish the correct diagnosis. Without proper clinical diagnosis there is the possibility of a misdiagnosis leading to erroneous or absent genetic testing results, as in the case of Oguchi disease in which the identification of a *GRK1* mutation led to a re-appraisal of the clinical phenotype that was previously incorrectly established as retinitis pigmentosa (RP).[3]

The next step after successfully analyzing the clinical phenotypes is the selection of a suitable strategy to find the causative genetic defect, which in our study was homozygosity mapping in most of the families. In addition below I will discuss the power of homozygosity mapping in identifying the mutations in consanguineous and non-consanguineous families.

8.2. Homozygosity mapping as a powerful tool for research and diagnosis

This thesis describes homozygosity mapping analysis of 26 Pakistani families, 16 (61.5%) of which were found to have mutations in genes previously associated with retinal diseases including 6 novel and 10 previously discovered mutations (**chapters 2, 3, 5 and 6**). Two large consanguineous families with RP described in the **second chapter** had mutations in *TULP1*, one mutation has previously been reported in the Pakistani population whereas the second mutation was novel. In silico analysis predicted these mutations as pathogenic. In the **third chapter** two consanguineous FA families have been described to have two novel mutations in *RDH5*. Patients of these two families are potential candidates for oral supplementation of 9-*cis* beta carotene. In the **fifth chapter** four consanguineous families have been described. In one family a novel missense mutation was identified in *PDE6A* whereas in the remaining three families previously identified protein truncating mutations were detected in *ABCA4*, *AIPL1* and *CERKL*. The sixth chapter describes a total of 17 families, of which mutations in known retinal disease genes were detected in 8 families whereas 9 families remained unsolved. In 6 of the 9 unsolved families we have excluded the presence of mutations in genes previously implicated in autosomal recessive RP or LCA that were present in the homozygous region. In 3 other families there were no genes previously implicated in inherited retinal diseases in the outstanding homozygous regions. Therefore, in these 9 families there is a

possibility of identifying mutations in genes not yet implicated in these diseases. However, the presence of compound heterozygous mutations as well as deep intronic and promoter region mutations in genes previously implicated in inherited retinal diseases cannot be ruled out. In one family a novel mutation was identified in a gene (*DHX38*) that hitherto has not been implicated in autosomal recessive RP (**chapter 7**).

Similar to a number of previous studies describing the power of homozygosity mapping in the identification of disease causing genes in consanguineous families,[3-7] we were also able to identify the genetic defect in most of our consanguineous families. Similarly, this approach can also be used to detect disease causing mutations in non-consanguineous families,[4,8-10] which is evident from the resolution of the two non-consanguineous RP families described in the **sixth chapter**, in which mutations were identified in *CNGA1* and *RPE65*.

Using homozygosity mapping mutations were detected in 61.5% of families included in this thesis. In a previous study 30% of Leber congenital amaurosis (LCA) families were solved using homozygosity mapping.[4] This difference indicates that the Pakistani population has higher potential to be used for mutation detection in known retinal disease genes using homozygosity mapping as compared to other populations. Similarly, homozygosity mapping is equally beneficial for mutation detection in outbred families as evident from the results of two solved families with mutations in *CNGA1* and *RPE65*.

Therefore, from this thesis it is evident that homozygosity mapping is a powerful tool to identify mutations in genes known to be implicated in inherited retinal disease, but for the identification of genes not previously associated with these diseases, this technique in most cases needs to be followed by exome sequencing.[11-14] However, when an obvious candidate gene associated with a retinal phenotype in animal models is present in the identified homozygous region it can be screened for causative mutations.[15-19]

8.3. The use of exome sequencing in molecular diagnosis

In this thesis, exome sequencing proved to be a powerful technique for the identification of novel as well as previously identified mutations in known disease-causing genes. In addition exome sequencing successfully identified a potential disease causing mutation in a novel gene, *DHX38*, in a family with severe early-onset RP.

In the **fourth chapter** two BBS families have been described with a novel splice donor site mutation and a previously identified mutation in *BBS1*, that were identified through exome

sequencing. Affected individuals of both families were diagnosed with BBS. In the patients of family A, intellectual disability was not present whereas in family B, patients did show intellectual disability. Another notable observation was the presence of polydactyly in the affected female but not the affected male of family A. Although exome sequencing was performed, this intrafamilial phenotypic variability that is mostly attributed to unknown modifiers remained unsolved and needs further analysis. Similarly, *in silico* analysis indicated that both mutations identified in *BBS1* are likely pathogenic but *in vitro* studies as well as *in vivo* studies in model organism are needed to confirm the results of *in silico* analysis.

In another consanguineous family (**seventh chapter**) with severe early-onset RP, homozygosity mapping was followed by exome sequencing, which resulted in the identification of a missense mutation in a novel putative disease causing gene, *DHX38*. Since to date, no second mutation in *DHX38* has been described to be causative for RP, there is as yet no conclusive proof that *DHX38* variants underlie retinal degeneration. In this family initial homozygosity mapping aided in the narrowing down of a huge list of variants that were identified in exome sequencing, to prioritize the variants for segregation analysis. In addition, uncovered exons of known retinal disease genes were excluded by haplotype comparison that was not possible in the absence of homozygosity mapping data.

Based on these findings, exome sequencing has the potential to be used in the Pakistani population for the molecular diagnosis of inherited eye diseases as well as other commonly inherited diseases like deafness, microcephaly, familial hypercholesterolemia etc. But for the identification of novel disease causing genes homozygosity mapping has its own advantage in combination with exome sequencing. The Pakistani population has a high rate of consanguineous marriages; therefore, homozygosity mapping can be used as an initial step before proceeding to the exome sequencing. Currently, an exome sequencing facility is not available in Pakistan.

As homozygosity mapping is a powerful technique in the identification of disease-causing loci/genes and exome sequencing is a comparatively fast and cheap method, a future systematic approach that can be used for the genetic screening of Pakistani patients and families with inherited retinal as well as other inherited disorders has been proposed in Figure 8.1. In a molecular diagnostic setup, exome sequencing, followed by bioinformatic analysis focusing on the known retinal disease genes, may lead to the identification of the disease

causing mutations in more than 60% of the patients. In case of exclusion of the known genes, a discovery of novel genes might require further research.

Exome sequencing can lead to three different possibilities (Figure 8.1). One possibility is the detection of homozygous or compound heterozygous protein-truncating mutations in the genes that are highly expressed in the retina or involved in a retinal pathway. The second possibility is the identification of homozygous or compound heterozygous missense mutations in retinal genes. There is a need to optimize a protocol for the evaluation of identified-variant pathogenicity to confirm the involvement of a particular variant. The third possibility is to filter out a causative mutation from a large number of variants identified in the exome sequencing. This last task is the most challenging one as it is difficult to prove the role of a large number of mutated genes in the retinal processes. Hence for this issue, homozygosity mapping and linkage analysis are of great value. Haplotype comparison and calculation of LOD-scores could lead to a valuable conclusion. In the future, it is hoped that pathogenicity assessment of most of the identified variants will be easily available but the selection of causative variants will still be a challenge in the presence of thousands of variants identified in the exome sequencing.

8.4. Allelic heterogeneity, phenotype variability, and the establishment of genotype-phenotype correlations

Inherited eye diseases generally exhibit clinical and genetic heterogeneity, the phenotypes can vary between individuals with the same or different genetic variants in the same gene.[1,20-23] Therefore, linking different genetic mutations to the exact phenotypes in affected individuals enhances the possibility of finding causative variants. Families described in the **second chapter** were found to carry *TULP1* mutations, which is a good example of making use of a genotype-phenotype correlation.[24] It had previously been observed that the formation of a perifoveal ring around the macula of affected individuals was characteristic of *TULP1* mutations.[4] During the initial diagnosis of the relevant families we did not notice the perifoveal ring. However, after performing the homozygosity mapping, and subsequent mutation detection in *TULP1*, we re-evaluated the clinical findings in the families, upon which the perifoveal rings were observed. This again highlights the need for a clinical diagnosis by an expert clinician who can critically evaluate the patient phenotypes.

The perifoveal ring was found to be present on the fundus of all the affected individuals in one family carrying *TULP1* mutations. However, in the other family this ring was incomplete,

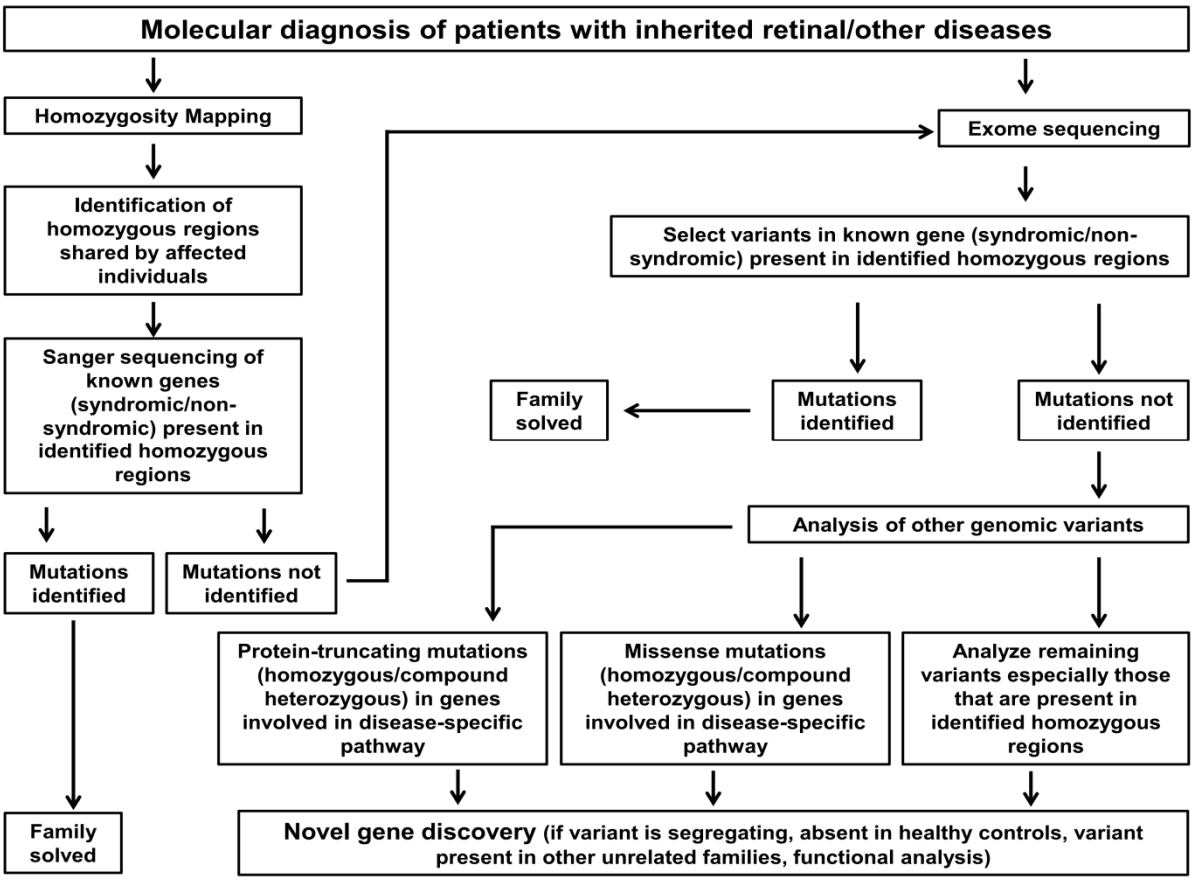

Figure 8.1 Flow chart showing proposed steps in the identification of genetic causes of inherited diseases in Pakistani population.

which suggests that when the perifoveal ring is seen on the fundus it is worthwhile to screen the *TULP1* gene before using other techniques, such as homozygosity mapping, linkage analysis or exome sequencing, thereby saving time and money.

In total three *TULP1* mutations, one novel (p.(Arg482Gln)) and two previously identified mutations (p.(Thr380Ala) and p.(Lys489Arg)) were identified in the current study.[25,26]

Families described in the **third chapter** are a good example of a genotype-phenotype correlation of *RDH5* mutations which are associated with typical white dots in the mid-periphery of the retina. Novel *RDH5* mutations (p.(Val305Hisfs*29) and p.(Met253Arg)) were identified in two consanguineous Pakistani families with FA patients.[27] The FA patients of family B carrying the same *RDH5* mutation and variable phenotypes exhibit clinical heterogeneity. As in the families with the *TULP1* mutations, families with *RDH5* mutations were initially subjected to SNP microarray analysis without detailed clinical evaluation, which resulted in the identification of a homozygous region containing the *RDH5* gene in both families. Upon subsequent clinical evaluation of the affected individuals of both

families, they were diagnosed as FA patients.

An overview of all published *RDH5* mutations was analyzed to determine genotype-phenotype correlations. It was observed that the presence of white dots was the only shared clinical feature among persons with *RDH5* mutations.[27] Thus, it can be concluded that the presence of white dots on the fundus are characteristic of persons carrying *RDH5* mutations and that the initial screening of *RDH5* gene is mandatory in FA patients. Upon exclusion of *RDH5*, two other genes, *RLBP1*[28] and *RPE65,*[29] can be screened to determine the causative mutations. Another important finding was the presence of macular degeneration in elderly patients of both families, which might suggest a progressive course of disease. Follow-up fundus examination of younger patients who do not have macular degeneration at this stage is required and if they develop any signs of macular degeneration then this disease can be classified as RPA instead of FA.[2]

In the **fourth chapter** two Bardet-Biedl syndrome (BBS) families with *BBS1* mutations (c.47+1G>T and p.(Asp148Asn)) are described. Affected individuals of family A were found to have the same *BBS1* splice donor site mutation but the proband's sister also had polydactyly, which was not present in the proband. This intra-familial phenotype variability might be due to some other still unknown genetic modifier present in the family. This is also supported by the observation that in Bardet-Biedl syndrome (BBS) families, the effect of modifier alleles on the patient phenotype is well documented.[30] Affected individuals of both families had sufficient clinical features for assigning a BBS status. This points to our observation that in clearly diagnosed families genetic testing provides conclusive results.

In the **fifth chapter** four consanguineous RD families have been described with recurrent nonsense mutations in *ABCA4*, *AIPL1* and *CERKL*, and a novel missense mutation in *PDE6A*. A previously identified mutation p.(Gln2220*) in *ABCA4*[31] was identified in all affected individuals of the family.

A second previously identified nonsense mutation (p.(Trp278*)) was identified in *AIPL1* in a family with early-onset RP/LCA. *AIPL1* mutations are mostly associated with LCA[25,32,33] but we could not establish a correlation between this mutation and its associated phenotype, which can be further used for population screening to identify additional patients carrying this mutation.

A third previously identified nonsense mutation (p.(Arg283*)) was identified in *CERKL* in a family with RP. Previously this mutation was identified in Spanish patients.[34] Comparison of phenotypes did not reveal any link between the type of the mutation and the severity of the phenotype.

In the fourth family a novel mutation (p.(Arg544Trp)) was present in *PDE6A*. *In silico* analysis predicted that this mutation is likely to be pathogenic but like the above mentioned three families a genotype-phenotype correlation was not established.

In the **sixth chapter** a comprehensive overview of the retinal diseases in the Pakistani population highlights the association of different gene defects and corresponding phenotypes. A previously identified mutation (c.488-1G>A) in *IQCB1* was found in a family of affected individuals with retinal vessel attenuation in two affected brothers. Optic disc drusen were present in both eyes of the proband only. The youngest brother was diagnosed as RP *sine pigmento*. Although in the absence of any complaints these patients were not tested for renal defects, they are likely to develop renal malfunctions later in life, as *IQCB1* mutations are known to be associated with nephronophthisis.[35] Future follow-up studies are needed to confirm the severity of the disease in this family.

Mutations in other retinal disease-associated genes such as *NR2E3* and *TULP1* have also been reported to cause phenotype variability in patients belonging to the same as well as different families, and this variability is thought to be a result of modifying effects of unknown trans-acting alleles.[22,23] A modifying effect of variants in *RPGRIP1L* (p.(Arg744Gln)) and *IQCB1* (p.(Ile393Asn)) have previously been reported in X-linked RP patients with *RPGR* mutations.[36]

Two recurrent *RPE65* mutations (p.(Arg44Gln) and p.(Ser121Leufs*5)) were identified in affected members of two families. Mutations in this gene are known to be associated with recessive and dominant forms of RP, as well as autosomal recessive forms of Leber congenital amaurosis,[37-42] Very recently, compound heterozygous mutations in *RPE65* have been reported in FA.[29] Due to this phenotypic variability it is difficult to establish a conclusive relationship between identified mutations and associated phenotypes for these two families with *RPE65* mutations.

CNGB1 and CNGA1 are rod specific proteins and are subunits of the cyclic nucleotide gated (CNG) channel. In this thesis a novel *CNGB1* splice site mutation, c.2493-2A>G, was

identified in a large consanguineous family. Previously, *CNGB1* mutations have been reported to cause RP in families of different ethnic origins.[43,44] Currently, as there is no established genotype-phenotype correlation for *CNGB1* mutations, we were thus unable to establish any significant relationship between genotype and phenotype of the affected individuals. Another novel mutation was found in *CNGA1* (c.1298G>A; p.(Gly433Asp)) in a family with RP patients. Mutations in this gene are also known to cause RP in a few families including one Pakistani family.[45,46] As in persons with *RPE65* and *CNGB1* variants there are no significant differences in the phenotype of patients with *CNGA1* mutations as compared to the phenotype of patients with mutations in other genes.

A common recurrent mutation, p.(Thr745Met), in *CRB1* was found to be the underlying genetic defect in another family in this study. This variant has been found in persons with LCA, early-onset RP and classical RP, but no clear genotype-phenotype correlation has been established previously.[47-49]

In all families in which only males were affected, haplotype analysis of the *RPGR* and *RP2* containing regions of the X-chromosome could not exclude their involvement. We sequenced *RPGR* and found a previously identified *RPGR* mutation, p.(Glu809Glyfs*25),[50] in one family. Clinical data of this family were not available; therefore, follow up examination is needed to investigate any genotype-phenotype correlation.

In the **seventh chapter**, a novel candidate gene implicated in severe early-onset RP has been described. In a consanguineous family, genome-wide homozygosity mapping identified a single homozygous region, where there were no previously implicated genes in this region. Upon exome sequencing analysis of the proband, a potentially pathogenic homozygous missense variant (p.(Gly332Asp)) was identified in *DHX38*, that segregated in this family. DHX38 is also known as PRP16 and is involved in the catalysis of the second step in pre-mRNA splicing. Previously, splicing factor gene mutations have been shown to cause autosomal dominant RP. In the current study we hypothesize the involvement of *DHX38* in autosomal recessive RP. In addition, this mutation was not found to be present in ethnically matched controls. Sequence analysis was conducted of DNAs from other inherited retinal disease families with outstanding homozygous regions encompassing *DHX38*, but we did not find any other mutation in *DHX38*. However, the high evolutionary conservation of the mutated nucleotide and amino acid residue, the absence of this variant in 180 ethnically matched control individuals, its presence in the largest (10.9 Mb) homozygous region, and the

absence of other variants in exome sequencing, argue that *DHX38* is a plausible rare genetic cause of severe early-onset RP. But, further studies are needed to prove the retinal pathogenicity of mutations in *DHX38*.

One of the main objectives of the current thesis was to set a foundation towards the treatment of inherited eye diseases based on genetic knowledge. Therefore in the following section I will discuss different possibilities of therapeutic interventions for the individuals with inherited retinal diseases in which the causative variants have been identified.

8.5. Possibility of therapeutic intervention

The identification of causal DNA variants is the first step towards the treatment of inherited retinal diseases. Based on the type of mutations some of the inherited eye diseases can be prevented by oral supplementations, such as the treatment of FA with 9-*cis* beta carotene.[51] In our study the affected individuals of two families with *RDH5* mutations are good candidates for 9-*cis* beta carotene therapy.[27]

Another treatment option is available for individuals with *RPE65* mutations as the underlying genetic cause of the disease.[52-56] *RPE65* gene therapy trials have proven to be successful in human subjects, which involve the delivery of a recombinant vector containing a normal copy of *RPE65* to RPE cells. It has been suggested that *RPE65* gene therapy is more effective in children as compared to adults,[52] as in the latter the disease has progressed to a stage where gene therapy is ineffective. Besides this, gene therapy patients with *RPE65* mutations can benefit from the most recently developed oral drug QLT091001, which is a synthetic *cis* retinoid. Patients who received QLT091001 therapy were found to have significant visual improvements (http://clinicaltrials.gov/ct2/home).

In this thesis affected individuals of two families were diagnosed with *RPE65* mutations. The main hurdle to implement gene therapy in these patients was the lack of expertise. In addition, follow up examinations are also required to detect the efficacy of the injected vector. Therefore if the patients are to benefit from this intervention it is imperative to establish a cost-effective and easily accessible gene therapy facility in Pakistan.

Based on the above findings different treatment options can be considered for the patients. For this purpose the establishment of a strong link is required between clinicians and geneticists. Below I discuss the need for establishing a functional link between these two important entities of genetic research.

8.6. Linking clinicians and geneticists

One of the major objectives of the current research was to establish a strong link between the clinical practice and genetic testing by spreading the knowledge of molecular genetics and emphasizing the importance of identifying the genetic defects in affected individuals with inherited eye disorders in Pakistan. This objective is not achievable without the consent of practicing clinicians as they are the main contact persons for the patients in the hospitals. Geneticists can help the clinicians by identifying the genetic causes, and the clinicians can implement the results of genetic testing by providing genetic counseling and treating the patients with available therapies.

Exploration of the gene based molecular therapeutic options by clinicians can go a long way in the improvement of the society. Although the molecular therapeutics field is an emerging field it will take several years before treatments have passed the trial phases and will become routinely available. Overall, it is worth to mention that the current experience with gene therapy is that it takes at least ten years from gene discovery to first clinical trial, and at least another five years for going through these trials. In the mean time, clinicians with knowledge of the molecular basis of the patients' diseases may be able to satisfy the psychological needs of the patients.

The following section is dedicated to the discussion of possible benefits to the participating patients and their families.

8.7. Benefits to the patients and families

The work presented in this thesis will be beneficial to the participating affected and healthy individuals in many ways. Firstly, affected individuals can benefit from readily available therapeutic options (oral supplementations etc.). In our study two families with *RDH5* mutations may benefit from oral supplementation of 9-*cis* beta carotene. Secondly, genetic counseling of persons carrying a mutation in a heterozygous or homozygous state may guide them to select their future spouses to prevent the disease from spreading in the family. These two benefits are the foremost options which can be immediately implemented. Another direct benefit to the patients is the monitoring of their disease progression by follow up examinations, which might be beneficial in slowing down the disease progression.

Based on the current knowledge, only a limited number of persons with retinal diseases would be amenable for the currently available gene therapy trials, although due to cost issues most of

the patients residing in the developing countries are still far from benefitting from this option. Therefore, in this situation, genetic counseling remains the only option for people who have affected siblings or other near relatives. Proper counseling and documentation of the disease based on the genetic testing may aid in the correct diagnosis,[57] without which sometimes even expert clinicians may misdiagnose a disease. Patients with inherited disorders often become hopeless when they keep on visiting a number of health care providers and are unable to receive proper therapeutic intervention or advice.

In this thesis, we identified a mutation in *IQCB1* (**sixth chapter**) in a consanguineous family, which has been implicated in Senior-Loken syndrome. Patients with *IQCB1* mutations often experience kidney malfunction later in life.[58,59] Identification of an *IQCB1* mutation in this family allows a detailed monitoring of renal function, and as a result, proper management is possible in order to delay the disease progression, which had not been possible without this molecular evidence. In families with mutations in *BBS1*, *CNGA1*, *CNGB1*, *CRB1*, *RPGR* and *TULP1* genetic counseling at this moment is the only option as currently there is no oral supplementation or gene based therapy available for patients with mutations in these genes.

Similarly, a family described in the **seventh chapter** with a *DHX38* variant has the only option of genetic counseling to prevent the spread of the disease in the next generations. We still need to keep in mind that the *DHX38* variants have not been proven beyond doubt that they are causative. Another benefit of such research is that affected persons gain knowledge of the molecular basis of their disease. This option is not very helpful for illiterate patients, but well educated relatives might help to educate them about the disease and its genetic etiology. With the spread of genetic knowledge there is the possibility of establishing patient support groups that in future may aid in raising funds for the development of therapies for inherited eye diseases.

8.8. Future research directions

Genetically, inherited eye diseases like many other genetic disorders are a great socioeconomic burden on the families with single or multiple affected individuals, as they cannot cope with their social liabilities and always remain dependent on their families. This is especially so in Pakistan, where the disability of affected individuals exerts extreme psychological pressure on them. But with the advent of modern molecular biology techniques, modern research has begun to unravel the molecular causes of human diseases, which has now lit a candle in the dark for such patients and their families, who are enthusiastically

taking part in such genetic screenings.

Currently, the whole field of molecular genetic research have seen a revolution and new methods are continuously being developed that are faster, more accurate and cost effective. However, this revolution has still not improved the patients' quality of life from the genetic testing perspective, and the patient is still far from benefiting from the available genetic therapies. Hence, there is a need to streamline each and every step of genetic research; the first and foremost thing in this regard is the establishment of a central facility to enroll patients with inherited eye disease for which currently no treatment is available. The next step would be the genetic testing, which is already being done at different laboratories in Pakistan. Another important aspect of the future research direction is the development of a cost-effective method for the screening of 13 most commonly inherited mutations **(sixth chapter)** that are prevalent in the Pakistani population. An arrayed-primer extension (APEX) microarray may be the most suitable and cost-effective method for screening purposes. This method may identify more than 40% of the genetic causes of inherited retinal dystrophies. Although APEX will become obsolete within the next few years and most probably exome sequencing and whole genome sequencing in combination with most advanced techniques like ion torrent and nano pore technology will completely take over, this can at least bridge the gap till the spread of new technologies.

Further, there is a need to suitably convey the outcome of the genetic testing to the family. For this purpose a central patient enrollment facility should be advised to arrange genetic counseling experts, who can then advise the families and patients.

Another important issue is the financial situation of the patient, which should always be kept in mind. Establishment of a transparent fund raising agency for genetic testing is likely to solve this issue though it is a difficult step. After these issues have been addressed, we can proceed further in helping the affected individuals. Although in countries like Pakistan there is a shortage of state-of-the-art facilities, it is still possible to solve these issues by establishing strong connections with international research organizations that can provide access to their facilities. Professionals will need to continue their efforts to convince the patients as well as clinicians to participate in genetic screening studies.

One important issue, which needs to be addressed in the future, is the pathogenicity assignment to different mutations identified in affected individuals of different families. Currently, in most of the studies and in this thesis pathogenicity is assessed using *in silico*

tools. In addition, segregation analysis and absence of variations in healthy controls are used to confirm the pathogenic status of different mutations in genes. With the advent of exome sequencing techniques, the number of identified variants has increased tremendously. This situation has further complicated the issue of pathogenicity assessment. In the current scenario people are concentrating on those variations that are present in the identified homozygous regions (in consanguineous families) or compound heterozygous variants (in outbred families) and potentially pathogenic according to the *in silico* tools.

Finally, we successfully solved 19 (67.8%) families using homozygosity mapping and exome sequencing combined with Sanger sequencing. In 9 (32.2%) families, exclusion of known loci indicated the presence of novel disease causing genes.

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Summary

Summary

A total of 26 consanguineous and 2 non-consanguineous families with inherited eye diseases and multiple affected siblings were included in the current study. With one exception, homozygosity mapping using single nucleotide polymorphism (SNP) arrays was used to find genomic regions potentially carrying the mutated genes in the families. In two families, exome sequencing was performed directly without first conducting SNP microarray analysis. This allowed us to benefit directly from this technology and also allowed us to assess the power of exome sequencing in the diagnosis of inherited disorders. In 18 families, mutations were identified in genes previously implicated in inherited retinal diseases. Our main focus remained on autosomal recessive forms of retinitis pigmentosa (arRP), fundus albipunctatus (FA), and Bardet-Biedl syndrome (BBS). In one family, a causative variant was found in the X-linked *RPGR* gene. In the remaining nine families we could not identify causal genes.

In the general introduction (**first chapter**), a brief anatomy of the human eye including the retina and photoreceptors was described. The mechanism of the phototransduction cascade and the retinoid cycle were also explained in detail. Three types of inherited eye diseases, i.e. RP, FA and BBS, were introduced. Different techniques used in the identification of genetic defects were described briefly as well as the currently available treatment and prevention options for persons with inherited eye diseases.

Chapter 2 describes an early-onset form of autosomal recessive early-onset RP in two large consanguineous families, in which recurrent and novel mutations in *TULP1* were the underlying genetic cause. TULP1 belongs to the tubby like family of proteins and is exclusively expressed in retina, where it takes part in the transport of rhodopsin from the photoreceptor inner segment to the outer segment. The p.(Thr380Ala) variant identified in one of the present families was previously identified in two Pakistani families. Identification of the same mutation in a large family with multiple affected siblings in the current study highlights the possibility of a Pakistani founder allele. The second mutation identified in *TULP1,* p.(Arg482Gln), was novel, and likely affects the tubby domain of TULP1. Three dimensional protein structure modeling also predicted structural modification that may affect the normal function of the protein, ultimately impairing vision.

The **third chapter** is dedicated to another form of retinal disease known as autosomal recessive FA. In this chapter two novel mutations were identified in *RDH5* (p.(Val305Hisfs*29) and p.(Met253Arg)) to be the cause of FA. *RDH5* is predominantly

expressed in the retinal pigment epithelium (RPE) where the encoded protein is an important part of the visual cycle, involved in the regeneration of the chromophore 11-*cis* retinal. Through collecting all published *RDH5* variants, this chapter also investigated genotype-phenotype correlations for *RDH5*. Mutations in this gene were found exclusively in persons with white dots on the fundus. Thus, the presence of white dots on the fundus suggests an initial sequence analysis of *RDH5*, followed by the analysis of *RLBP1* and *RPE65*, two genes that have also been associated with FA. Another important finding of the current study was the presence of macular degeneration in elderly patients of both families, which may suggest the progressive nature of the disease. Follow up fundus examination of younger patients without macular degeneration is mandatory and if they show any signs of developing macular degeneration then that might confirm the diagnosis of retinitis punctata albescens (RPA) instead of FA in these families.

In the **fourth chapter**, two families with BBS have been described in which two different *BBS1* mutations (c.47+1G>T and p.(Asp148Asn)) were identified. BBS is a syndromic form of rod-cone dystrophy and most of the genetic subtypes are considered ciliopathies. The presence of severe phenotypes in both families contradicts the conclusions of a previous study which found the *BBS1* mutations to have a less severe phenotype as they had better visual acuities and better electrophysiological response of rods and cones (Daniels *et al*., Archives of Ophthalmology, 130, 901-907, 2012; Estrada-Cuzcano *et al*., Archives of Ophthalmology, 130, 1425-1432, 2012). Another finding of our study was the intra-familial phenotypic variability in one family in which the affected sister had polydactyly whereas the affected brother did not. This probably can be attributed to the presence of unknown modifiers. This chapter also describes the suitability of exome sequencing for the diagnosis of inherited diseases in families where the disease can be caused by mutations in a number of different genes.

In the **fifth chapter**, four consanguineous families with retinal dystrophy have been described to have recurrent and novel mutations in four different genes, respectively. Previously described protein-truncating mutations were identified in *ABCA4*, *AIPL1* and *CERKL* in three families, whereas a novel missense mutation was identified in *PDE6A* in the fourth family. This chapter also demonstrates the power of homozygosity mapping in identifying the disease-causing mutations in consanguineous families.

The **sixth chapter** provides a comprehensive overview of the genetic causes of syndromic and non-syndromic inherited retinal diseases in Pakistan. In addition, the causal variants in genes previously implicated in inherited retinal diseases are documented for 8 different families. A novel *CNGA1* mutation (p.(Gly433Asp)) and a novel *CNGB1* mutation (c.2493-2A>G) were identified in two RP families, while in 6 other families mutations in *CRB1*, *IQCB1*, *RPE65*, *RPGR* and *TULP1* were identified that had been found previously. In addition, we also present the data of 9 families with homozygous regions lacking genes previously implicated in inherited retinal diseases or in which the latter genes were excluded to carry causative variants using Sanger sequencing. For these families LOD scores of homozygous regions ranged from 1.8 to 4.0. In two families highly suggestive linkage (LOD >2.7), was found whereas for two other families significant LOD scores (>3.3), were found. However, in one family a single homozygous region of 46.1 Mb was found on chromosome 1 (LOD score 2.7), which overlapped with the RP32 locus (Zhang *et al*., Human Genetics, 118, 356-365, 2005). The remaining five families contained multiple homozygous regions. Although we cannot exclude possibilities of deep intronic and compound heterozygous mutations as well as mutations in promoter regions of excluded genes in these families, there is a strong possibility of finding mutations in 'novel' disease causing genes, and exome sequencing analysis is currently ongoing for these families.

A consanguineous family described in the **seventh chapter** was found to have a novel mutation (p.(Gly332Asp)) in *DHX38*, which was shown to segregate with the disease in the family. *DHX38* mutations can be a novel cause of severe early-onset RP. Further, studies on additional families as well as functional assays in animal models may shed further light on the possibility of establishing a clear genotype-phenotype correlation in this family.

In the **eighth chapter**, results of the research presented in this thesis have been discussed from different perspectives. First of all the need for correct and timely clinical evaluation has been highlighted which can assist the choice of genetic testing. The heterogeneous genetic nature and phenotypic variability of inherited retinal eye disorders were also discussed. The power of homozygosity mapping in the identification of retinal diseases in consanguineous families has also clearly been demonstrated. Genotype-phenotype correlations have been shown to be a very useful aspect in genetic screening. Possible treatment and prevention options, benefit of this research to patients, and future aspects like facilitating the patients in managing the disease, improvement of screening protocols in terms of cost-effectiveness and robustness, and financial status of the patients were also discussed.

This research will hopefully set the foundation for the better management and prevention of inherited retinal diseases in Pakistani patients in the future and will be helpful in improving the quality of life of affected persons and their family members.

Samenvatting

Samenvatting

In totaal werden 26 consanguine en 2 niet-consanguine families met erfelijke oogziekten geïncludeerd in deze studie. Met uitzondering van één familie werd homozygotie mapping uitgevoerd bij deze families m.b.v. 'single nucleotide polymorphism' (SNP) arrays. Met deze techniek werden genomische gebieden gezocht die oorzakelijke mutaties bevatten. In twee families werd exome sequencing gedaan zonder dat hieraan een SNP microarray analyse vooraf ging. Hiermee konden wij direct deze nieuwe technologie benutten voor het stellen van een genetische diagnose. In 18 families werden mutaties gevonden in genen die eerder geassocieerd waren met erfelijke netvliesziekten. Onze aandacht ging vooral uit naar autosomaal recessieve vormen van retinitis pigmentosa (arRP), fundus albipunctatus (FA), en Bardet-Biedl syndroom (BBS). In één familie werd een oorzakelijke variant gevonden in het X-gebonden *RPGR* gen. In de overige 9 families werden geen oorzakelijke mutaties gevonden.

In de algemene inleiding (**Hoofdstuk 1**), wordt de anatomie van het menselijke oog beschreven met speciale aandacht voor het netvlies en de fotoreceptoren. De fototransductie cascade en de retinoid cyclus werden in detail beschreven. Drie types van erfelijke oogziekten, d.w.z. RP, FA en BBS, worden geïntroduceerd. De verschillende technieken die gebruikt worden voor het opsporen van genetische defecten worden gepresenteerd, als ook de momenteel beschikbare mogelijkheden voor behandeling en preventie voor personen met erfelijke oogziekten.

Hoofdstuk 2 beschrijft een vroeg optredende vorm van autosomaal recessieve RP in twee grote consanguine families, met eerder beschreven en nieuwe *TULP1* mutaties. TULP1 behoort tot de tubby-eiwitfamilie en komt exclusief tot expressie in het netvlies, waar het betrokken is bij het transport van rhodopsine van de binnenste naar de buitenste segmenten van de fotoreceptoren. De p.(Thr380Ala) variant die werd gevonden in één van de in deze studie onderzochte families was eerder geïdentificeerd in twee Pakistaanse families. Identificatie van dezelfde mutatie in een grote familie met meerdere aangedane broers en zussen ondersteunt het idee dat hier sprake kan zijn van een Pakistaanse mutatie die specifiek voorkomt in de Pakistaanse populatie. De tweede mutatie, gevonden in *TULP1,* p.(Arg482Gln), is nieuw en heeft waarschijnlijk een storend effect op het tubby domein van TULP1. Ook in een voorspelde 3-dimensionale eiwitstructuur van TULP1 heeft deze aminozuur substitutie een negatieve invloed op de functie van TULP1 eiwit.

Het **derde hoofdstuk** is gewijd aan een andere netvliesziekte, nl. autosomaal recessieve FA. In dit hoofdstuk worden twee nieuwe mutaties in *RDH5* (p.(Val305Hisfs*29) en p.(Met253Arg)) beschreven als oorzaak van FA. *RDH5* komt vooral tot expressie in het retinal pigment epitheel (RPE) waar het eiwit een belangrijke schakel is in de visuele cyclus, die zorgt voor de regeneratie van het chromofoor 11-*cis* retinal. Door middel van het verzamelen van alle gepubliceerde *RDH5* varianten, werd in dit hoofdstuk ook aandacht besteedt aan genotype-fenotype correlaties voor *RDH5*. Mutaties in dit gen werden exclusief gevonden in personen met witte stippen in de fundus. Indien personen witte stippen vertonen in de fundus ligt het voor de hand om eerst met sequentie analyse *RDH5* te onderzoeken, gevolgd door de analyse van *RLBP1* en *RPE65*, twee genen die ook werden geassocieerd met FA. Een andere belangrijke bevinding in deze studie was de aanwezigheid van macula degeneratie in oudere patiënten van beide families, hetgeen wijst op een progressief karakter van de ziekte. Het is zinvol om de fundus van jonge patiënten zonder macula degeneratie regelmatig te onderzoeken. Indien macula defecten worden gevonden, dan verandert de diagnose van FA naar retinitis punctata albescens in deze families.

In het **vierde hoofdstuk**, worden twee families met BBS beschreven met twee verschillende *BBS1* mutaties (c.47+1G>T en p.(Asp148Asn)). BBS is een syndromale vorm van staaf-kegel dystrofie en de meeste genetische subtypes worden beschouwd als ciliopathieën. De ernstige fenotypes die geconstateerd werden in beide families spreken de conclusies tegen van een eerdere studie waarin *BBS1* mutaties juist waren geassocieerd met een minder ernstig fenotype. De aangedane personen in deze andere studies vertoonden een betere gezichtsscherpte en ERG waarden electrofysiologische metingen van de staafjes en kegeltjes (Daniels *et al*., Archives of Ophthalmology, 130, 901-907, 2012; Estrada-Cuzcano *et al*., Archives of Ophthalmology, 130, 1425-1432, 2012). Een andere bevinding in onze BBS familie was de intrafamiliaire fenotypische variabilititeit omdat een aangedane vrouw polydactylie vertoonde en haar aangedane broer niet. Waarschijnlijk spelen onbekende 'modifiers' hierbij een rol. In dit hoofdstuk wordt ook de bruikbaarheid van exome sequencing beschreven voor de diagnose van erfelijke ziekten waarbij mutaties in verschillende genen betrokken kunnen zijn.

In het **vijfde hoofdstuk** worden 4 consanguine families met retina dystrofie bestudeerd met eerder gevonden en nieuwe mutaties in vier verschillende genen. Eerder beschreven mutaties die tot de afwezigheid van een functioneel eiwit leiden werden gevonden in *ABCA4*, *AIPL1* en *CERKL* in 3 families, terwijl een nieuwe missense mutatie werd gevonden in *PDE6A* in een 4^{e}

familie. Deze bevindingen illustreren de kracht van homozygotie mapping voor het vinden van de oorzakelijke mutaties in consanguine families.

Het **zesde hoofdstuk** geeft een compleet overzicht van de genetische oorzaken van syndromale en niet-syndromale erfelijke netvliesziekten in Pakistan. Tevens werden in 8 nieuwe families oorzakelijke varianten beschreven in genen die eerder werden geassocieerd met erfelijke netvliesziekten. Een nieuwe *CNGA1* mutatie (p.(Gly433Asp)) en een niet eerder beschreven *CNGB1* mutatie (c.2493-2A>G) werden gevonden in 2 RP families, terwijl in 6 andere families eerder gepubliceerde mutaties werden geïdentificeerd in *CRB1*, *IQCB1*, *RPE65*, *RPGR* en *TULP1*. Tevens werden gegevens van 9 families gepresenteerd waarin m.b.v. SNP microarray analyses homozygote gebieden werden aangetoond. In sommige gebieden werden genen gevonden die geassocieerd zijn met erfelijke netvliesziekten, maar werden geen mutaties gevonden m.b.v. Sanger sequencing. In andere families omvatten de homozygote gebieden geen bekende 'erfelijke netvlies ziektegenen'. In deze families varieerden de LOD scores van de homozygote gebieden tussen de 1.8 en 4.0. In 2 families werd zeer waarschijnlijke genetische koppeling (>2.7) gevonden, terwijl er in 2 andere families significante LOD scores (>3.3) werden aangetoond. In één familie werd een enkel homozygoot gebied van 46.1 Mb gevonden op chromosoom 1 (LOD score 2.7), welke overlap vertoonde met het RP32 locus (Zhang *et al*., Human Genetics, 118, 356-365, 2005). De overige 5 families droegen meerdere homozygote gebieden. Hoewel we diep-intronische, samengestelde heterozygote mutaties, en promoter mutaties niet kunnen uitsluiten in deze families, is er een gerede kans dat wij m.b.v. exome sequencing mutaties kunnen vinden in genen die tot dusver niet geassocieerd zijn met erfelijke netvliesziekten.

In een consanguine familie beschreven in **hoofdstuk zeven** wordt een nieuwe mutatie (p.(Gly332Asp)) gevonden in *DHX38*, welke segregeerd met de ziekte in de familie. *DHX38* mutaties kunnen een nieuwe oorzaak zijn van vroeg optredende RP. Vervolgstudies in andere families alsmede experimenten in diermodellen zullen definitief bewijs moeten leveren voor wat betreft de oorzakelijkheid van de gevonden varianten in dit gen.

In het **achtste hoofdstuk** worden de resultaten van dit proefschrift bediscussieerd. Ten eerste wordt het belang onderstreept van een correcte en tijdige klinische evaluatie, waardoor een afgewogen keuze gemaakt kan worden voor de uit te voeren genetische test. De genetische heterogeniteit en de fenotypische variabiliteit van erfelijke netvliesaandoeningen werden besproken. De kracht van homozygotie kartering in de identificatie van genen betrokken bij

netvliesziekten in consanguine families werd duidelijk aangetoond. Genotype-fenotype correlaties hebben hun waarde bewezen bij de genetische screening. Verder werd aandacht gegeven aan de mogelijkheden voor behandeling en preventie, het voordeel van dit onderzoek voor patiënten, het verbeteren van de screening protocollen wat betreft kostenefficiëntie en robuustheid, als ook de financiële status van de patiënten.

Dit onderzoek vormt de basis voor een betere toekomstige zorg en preventie van erfelijke retina ziekten in Pakistaanse patiënten en draagt bij aan het verbeteren van de kwaliteit van leven voor aangedane personen en hun familieleden.

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List of publications

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About the author

Muhammad Ajmal was born on Wednesday December 31, 1975 in Gujrat, Pakistan. He finished his school education in 1991 at the Deneys School, Rawalpindi. He did his Bachelor of Science degree from Gordon College, Rawalpindi in 1995. He enrolled for his Master of Science degree in Biochemistry in 1999 at the University of Arid Agriculture Rawalpindi and graduated in 2001. Then he joined Quaid-i-Azam University, Islamabad to complete his M. Phil degree in Biochemistry/Molecular Biology, where he worked on the identification of deafness genes in the Pakistani population under the supervision of Prof. Dr. Wasim Ahmad. After finishing M. Phil in 2005, Ajmal joined PCR Laboratories, Shifa College of Medicine, Islamabad, and worked on the molecular diagnosis of human pathogens. After two year (in 2007) he started his doctoral degree in molecular genetics at the COMSATS Institute of Information Technology, Islamabad, under the supervision of Prof. Dr. Raheel Qamar. During this study Ajmal was awarded a scholarship from Higher Education Commission (HEC) of Pakistan to conduct part of his research work in Nijmegen under the supervision of Prof. Dr. Frans Cremers and Dr. Rob Collin, where he worked on blindness genetics and enabled him to receive a joint doctoral degree from both universities.

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MUHAMMAD AJMAL

List of abbreviations

AA	amino acid
AAVs	adeno-associated viruses
ACHM	achromatopsia
ad	autosomal dominant
APEX	arrayed primer extension
ar	autosomal recessive
ARV	attenuated retinal vessels
BAM	binary alignment/map format
BBS	Bardet-Biedl syndrome
BE	bull's eye
BSP	bone spicule pigmentation
CB	cell bodies
CC	connecting cilium
CD	cone dystrophy
CF	counting fingers
cGMP	cyclic guanosine monophosphate
cGMP-PDE	cyclic guanosine monophosphate-phosphodiesterase
Chr	chromosome
CNG	cyclic nucleotide gated
CNVs	copy number variants
CRD	cone-rod dystrophy
CSNB	congenital stationary night blindness
DNA	deoxyribonucleic acid
DWD	deep whitish dots
DWF	deep whitish flecks
EORD	early onset retinal dystrophy
EORP	early onset retinitis pigmentosa
ERG	electroretinography
EVS	exome variant server
FA	fundus albipunctatus
Freq	frequency
GC	guanylyl cyclase
GCAP	guanylyl cyclase activating protein
GDP	guanosine diphosphate
GS	Grantham score
GTP	guanosine triphosphate
HM	hypermetropia
HGMD	human gene mutation database
Hz	homozygous
id	identifier
IS	inner segment
ISCEV	International Society for Clinical Electrophysiology of Vision
JBTS	Joubert syndrome
LCA	Leber congenital amaurosis
LE	left eye
LHM	low hypermetropia
LOD	logarithm of odds

LP	light perception
MA	macular atrophy
Mb	mega bases
MD	macular dystrophy
MKS	Meckel syndrome
NA	not applicable
NAD	nicotinamide adenine dinucleotide
NCBI	national centre for biotechnology information
ND	not determined
NGS	next generation sequencing
nm	nanometer
OMIM	online mendelian inheritance in man
OS	outer segments
PC	photoreceptor cell
PCR	polymerase chain reaction
PDE	phosphodiesterase
PhyloP	phylogenetic p-values
PolyPhen-2	polymorphism phenotyping v2
RE	right eye
RAL	retinal
RNA	ribonucleic acid
RD	retinal dystrophy
RetNet	retinal network
RFLP	restriction fragment length polymorphism
ROL	retinol
RP	retinitis pigmentosa
RPA	retinitis punctata albescens
RPE	retinal pigment epithelium
RPED	retinal pigment epithelium degeneration
SER	smooth endoplasmic reticulum
SIFT	sorting intolerant from tolerant
SLSN	Senior-Loken syndrome
SNP	single nucleotide polymorphism
SOLiD	sequencing by oligonucleotide ligation and detection
SRP	sectorial retinitis pigmentosa
USH	Usher syndrome
VA	visual acuity
VF	visual field
wt	wild-type
xl	X-linked